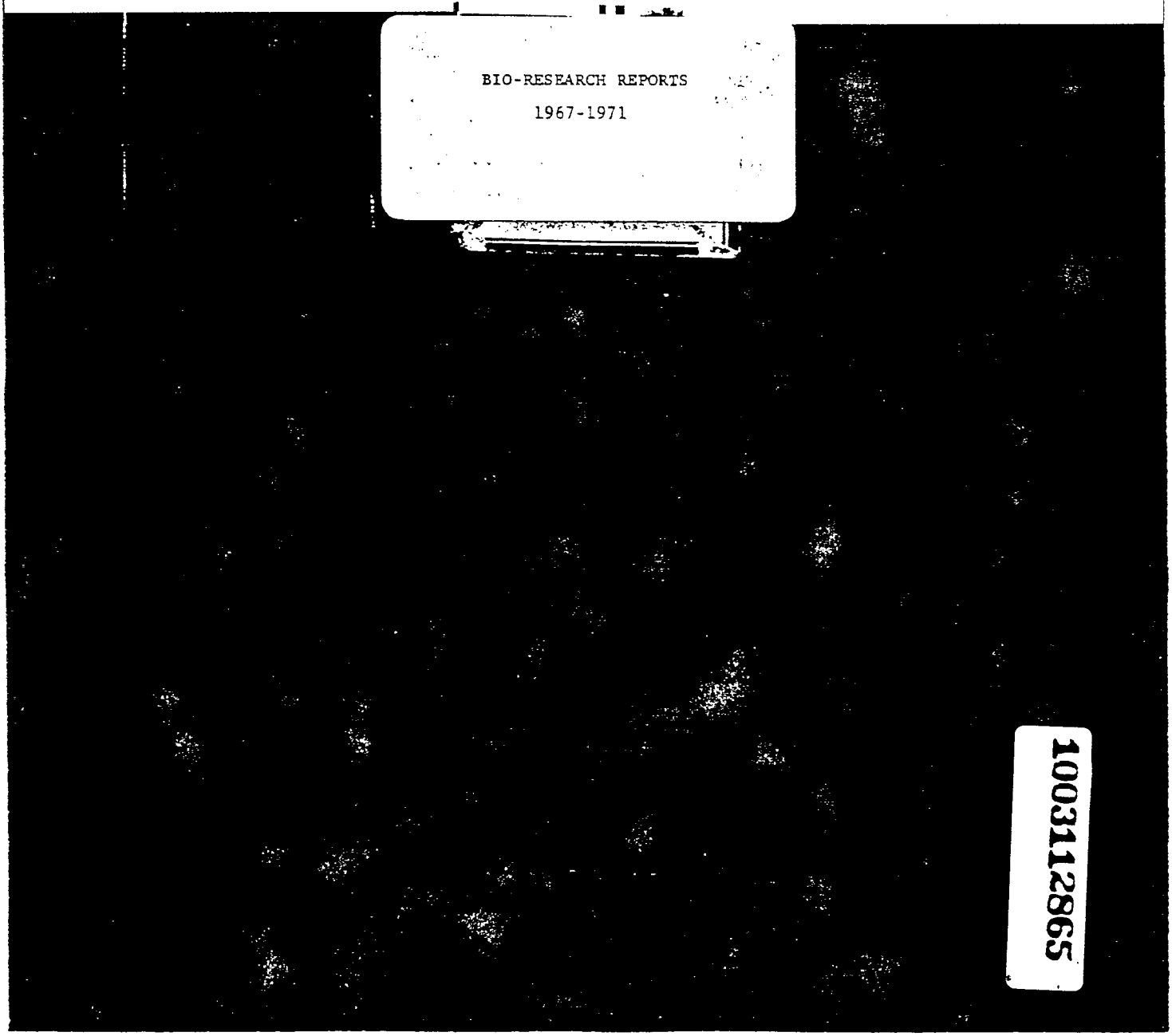

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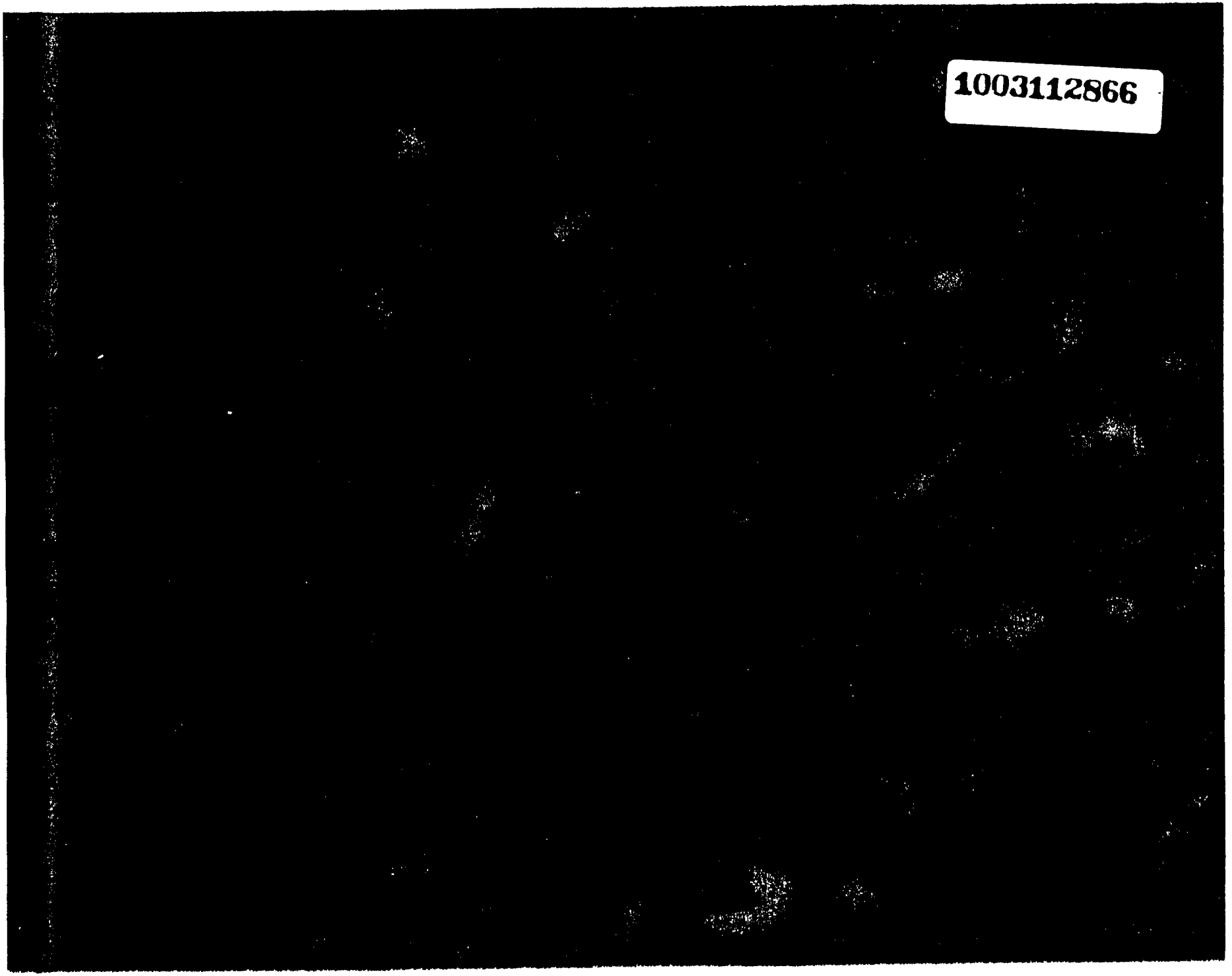
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BIO-RESEARCH CONSULTANTS, INC.
9 Commercial Avenue
Cambridge, Massachusetts 02141

R E P O R T

to

Report No. 24 (Final)

Contract C-172 (C-193)

Covering the period:

April 16, 1970 through April 30, 1970

Report Prepared by:

Cornelis G. Van Dongen, Ph.D., Research Associate

Distribution:

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Date August 16, 1971

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Bio-Research Consultants, Inc.

F-I-N-A-L R-E-P-O-R-T

The objective of Project C-172 was to develop a medicated cigarette. Medication would be incorporated into the cigarette and administration of this medication to the patient would be through inhalation. During the initial phases of C-172, a number of compounds were considered for the treatment of a variety of clinical problems, and several pilot studies were conducted prior to zeroing in on the specific objective -- to enrich cigarettes with atropine for the purpose of therapeutic use with clinical problems involving abnormally high glandular secretions. The clinical problems could theoretically be present in various areas, such as gastroenterology, sweating, salivation, and even reproduction.

The decision to work toward the development of an atropine-enriched cigarette was made primarily because of the facts that: 1) Atropine has been studied extensively from pharmacological and therapeutic standpoints. 2) Atropine is widely used in clinical practice. 3) Any new means of administration of atropine would inherit a broad range of potential applications. 4) The literature provides some qualitative experimental evidence with humans, suggesting that atropine can be transferred by cigarette whole smoke (Holmstedt, B. and Wallen, O.: Drug Administration by means of Cigarettes. Arch. Int. Pharmacol. 119: 273-293, 1959). This justifies that this experimental approach deserves careful quantitative studies.

Initially, the experiments should definitely not be done with humans. Aside from any legal problems, the cost of experiments with humans for this purpose would be prohibitive. It was therefore decided to do our experimental work with laboratory animals, preferably the least expensive ones, such as the mouse.

With regard to the selection of a physiological process, salivary secretion was chosen above any other of a variety of processes, parasympathetically controlled, because of the fact that it is the most sensitive one. This would allow the monitoring of even very small pharmacological dosages.

The two considerations stated above pointed to the need for an in vivo mouse biological assay which would use the salivary

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secretion process. An extensive search of the literature did not reveal the availability of such a method. We therefore developed an in vivo mouse bioassay whereby salivary secretion could be measured quantitatively on large numbers of nontraumatic, unanesthetized mice (a paper on this new methodology was presented at the fall meeting of the American Society for Pharmacology and Experimental Therapeutics in 1969, a copy of which is attached as Appendix No. 1 to this report). In addition to the technical aspects of the method, the paper presents quantitative information on the effects of various physiological conditions, such as sex, strain and individual, on salivary secretion.

Once the "normal" salivary performance of mice was established, we determined to what degree salivary secretion could be modified by chemical treatments. A broader data base of mouse salivation would thus be established for comparison with results in other species. Moreover, studies on the effects of atropine on the salivary secretion of the mouse would serve our specified objective. Appendix No. 2 consists of a paper on this subject which was presented at the annual meeting of the American Society for Pharmacology and Experimental Therapeutics in Atlantic City, N.J. in 1970.

The next logical step in this project appeared to be an extensive study of the effects of cigarette smoke on the salivary secretion of mice, which was carried out. The studies compared the effects of whole smoke vs. gas phases, various cigarette brands, and various smoke concentrations, from the low pharmacological through the high toxicological dose levels. The treatments were frequently tested on, not one, but two, different strains of mice or even on specified subgroups of individuals within a strain. These studies established that the salivary performance of mice in response to low cigarette smoke concentrations can be used as an indicator to estimate quantitatively the acute toxicity of these smokes at higher concentrations. Experimental data from these studies were presented at the annual meeting of the Society for Toxicology in Washington, D.C. (1971), and at the meeting of the American Industrial Hygiene Association in Toronto, Canada (1971). Copies of these papers are attached as Appendices III and IV.

This issue was raised: Are the effects on salivation, in response to exposure by cigarette smokes, due to the nicotine content of the various tobaccos? Our studies with nicotine on salivation led to the conclusion that the smoke effect on salivary performance can well be explained by the pharmacological action of nicotine alone. However, our studies do not exclude the possibility that factors other than

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nicotine might also be responsible. Only very carefully conducted quantitative additional studies might elucidate this. The effects of nicotine on salivary performance of two different strains of mice were reported at the FASEB meetings in Chicago (1971) (see Appendix No. V). Our nicotine studies also contributed to a basic understanding of the pharmacology of nicotine. We succeeded to define the required physiological conditions of the animal to obtain reproducible salivary responses with nicotine and we can now explain the controversial results of nicotine on salivation in the literature.

Finally, after Project C-172 had already expired, studies were conducted on our own to examine the effects of exposures to atropine-enriched cigarettes on the salivary performance of mice. Male C57BL/6 J mice (approximately 30 weeks old), which had been caged for some time while wearing collars, were used. These animals had been adapted to the salivation procedure and were all in good health. Their salivary performance was determined according to standardized procedures four times, with a one-hour interval between measurements. The first and second measurements established the control salivary performance of individual mice during that particular day. A ten-minute smoke exposure was applied immediately prior to the third measurement. The fourth measurement was performed to examine any possible aftereffect to the treatments. During the time interval between any two salivation measurements, the mice were returned to their "treatment" cages, which were supplied with adequate food and water.

The experimental cigarettes were Pall Mall, cut to 65 mm length. By means of a syringe with long needle, the cigarettes were homogeneously impregnated with 1 ml of absolute alcohol containing either 0, 0.4, 4.0 or 40 mg of atropine. The plain cigarettes and the four alcohol-treated groups of cigarettes were subsequently dried for one-half hour in a 40° C oven and then placed in a desiccator for approximately 18 hours, after which they were taken out for immediate use in the smoking machine.

The conditions of the smoking machine were standardized to 35 ml puffs of two seconds' duration, taken at intervals of 58 seconds. The smoke persisted for 15 seconds in the inhalation chamber and was expulsed in three seconds, followed by a 40-second fresh air purge. Teh puffs were used in each case, the first five puffs of each of two sets of cigarettes being smoked without interruption in the-one-puff-per-minute cycle. In order to obtain 10% smoke concentrations, we burned one experimental cigarette at a time.

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A single experiment started off with determination of the first and second salivation measurements on 72 mice. The sum of these two measurements served to rank order the mice according to salivary performance and allowed for any unexpected loss of mice. Next, 36 mice were assigned systematically to any one of six treatment groups (no exposure, exposure to whole smoke of Pall Mall, and exposure to the various atropine-enriched Pall Mall cigarettes). The remaining mice were eliminated from the experiment. Thus, the third and fourth salivation measurements were limited to a maximum of 36 mice (six mice in each of six treatment groups). The experiment was repeated once and the results of the two experiments were pooled into a set. A set of data was thus based on four measurements from 72 mice or, maximally, 12 mice per treatment group. One treatment, "machine exposure only," was not included, although it would have made the experiment more complete. The reasons were that it is very difficult to handle simultaneously seven treatment groups, and that earlier experiments had shown repeatedly that no significant differences existed between control and machine exposure when trained mice were used. When analyzing results, the 12 mice were separated into "high" or "low" salivators on the basis of their first and second salivation measurements. Using the Wang computing capability, we obtained significant data by means of established statistical techniques.

Results and Discussion

Tables No. 171 through 176 present the effects of the various treatments on salivary performance of C57BL mice (see also figures 35 through 39). There was dramatic salivary inhibition with any of the exposures of whole cigarette smoke. In addition, salivary inhibition by whole smoke was higher with the atropine-enriched cigarettes relative to that of their controls. Finally, it was shown that salivary inhibition is related to the atropine dose in a dose-response fashion. If substantiated by chemical experimental evidence, these results would then prove that the administration of atropine by means of inhalation is technically possible.

Conclusions: I. Strong experimental evidence is presented for the assumption that atropine is transferred by cigarette whole smokes.
II. The above-described experimental results indicate that the objectives of C-172 have been achieved.

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Potential Practical Applications for Mouse Salivation Bioassay:

1. Biological characterization of various cigarette brands;

2. Prediction of acute toxicity in cigarette smoke;

3. Testing of efficiency of various cigarette filters;

4. Estimation of degree of biological harmfulness of the particulate phase in whole cigarette smoke (still to be worked out);

5. Testing of the pleasurability of various additives to cigarettes (still to be worked out).

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Table 171

Mean Salivary Performances of Male C57BL Mice When Exposed to 10% Whole Smokes from Atropine-Enriched Pall Mall Cigarettes Immediately prior to the Third Salivation Measurement (Expressed in mm of boundary displacement)

Treatment	No. of Animals	Measurements			
		1	2	3	4
Control	11	56.6	69.0	64.4	46.2
Pall Mall plain	12	64.0	71.8	30.8	40.5
" " + alcohol	11	64.7	69.7	20.4	24.1
" " + 0.4 mg atropine	12	64.9	69.4	10.7	36.1
" " + 4.0 mg atropine	12	73.8	64.3	6.3	23.5
" " + 40.0 mg atropine	12	64.3	70.1	1.5	22.3

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Table 172

ANALYSIS OF VARIANCE
(Nonselected C57BL/6 J mice)

Measurement 1

Source	DF	SS	MS	F cal	F 95%	F 99%
Treatments	5	1,723	345	0.39	2.37	3.34
Error	64	55,962	874			
Total	69	57,685				

Measurement 2

Source	DF	SS	MS	F cal	F 95%	F 99%
Treatments	5	380	76	0.07	2.37	3.34
Error	64	72,030	1,125			
Total	69	72,410				

Measurement 3

Source	DF	SS	MS	F cal	F 95%	F 99%
Treatments	5	30,264	6,053	20.50**	2.37	3.34
Error	64	18,869	295			
Total	69	49,133				

** Significant at the 1% level of probability

Measurement 4

Source	DF	SS	MS	F cal	F 95%	F 99%
Treatments	5	5,956	1,191	1.98	2.37	3.34
Error	64	38,614	603			
Total	69	44,570				

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Table 173

Mean Salivary Performances of High-salivating Male C57BL Mice When Exposed to 10% Whole Smokes from Atropine-enriched Pall Mall Cigarettes Immediately Prior to the Third Salivation Measurement (Expressed in mm of boundary displacement)

Treatment	No. of Animals	Measurements			
		1	2	3	4
Control	5	85	86	67	48
Pall Mall, plain	6	85	105	31	48
Pall Mall + alcohol	5	83	97	27	23
Pall Mall + 0.4 mg atropine	6	87	86	8	27
Pall Mall + 4.0 mg atropine	6	95	83	5	30
Pall Mall + 40.0 mg atropine	6	77	98	1	22

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Table 174

ANALYSIS OF VARIANCE
(High-salivating C57BL/6J mice)

Measurement 1

Source	DF	SS	MS	F cal	F 95%	F 99%
Treatment	5	1,118	224	0.57	2.56	3.75
Error	28	10,943	391			
Total	33	12,061				

Measurement 2

Source	DF	SS	MS	F cal	F 95%	F 99%
Treatment	5	2,207	441	0.62	2.56	3.75
Error	28	19,895	711			
Total	33	22,102				

Measurement 3

Source	DF	SS	MS	F cal	F 95%	F 99%
Treatment	5	16,242	3,248	10.60**	2.56	3.75
Error	28	8,579	306			
Total	33	24,821				

** Significant at the 1% level of probability

Measurement 4

Source	DF	SS	MS	F cal	F 95%	F 99%
Treatment	5	3,983	797	1.25	2.56	3.75
Error	28	17,919	640			
Total	33	21,902				

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Table 175

Mean Salivary Performances of Low-salivating Male C57BL Mice When Exposed to 10% Whole Smokes from Atropine-enriched Pall Mall Cigarettes Immediately Prior to the Third Salivation Measurement (Expressed in mm of boundary displacement)

Treatment	No. of Animals	Measurements			
		1	2	3	4
Control	5	24	50	58	48
Pall Mall, plain	6	43	39	31	33
Pall Mall + alcohol	6	50	47	15	25
Pall Mall + 0.4 mg atropine	6	43	53	14	46
Pall Mall + 4.0 mg atropine	6	52	46	8	18
Pall Mall + 40.0 mg atropine	6	52	42	2	23

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Table 176

ANALYSIS OF VARIANCE
(Low-salivating C57BL/6 J mice)

Measurement 1

Source	DF	SS	MS	F cal	F 95%	F 99%
Treatment	5	3,039	608	1.27	2.55	3.73
Error	29	13,931	480			
Total	34	16,970				

Measurement 2

Source	DF	SS	MS	F cal	F 95%	F 99%
Treatment	5	758	152	0.36	2.55	3.73
Error	29	12,151	419			
Total	34	12,909				

Measurement 3

Source	DF	SS	MS	F cal	F 95%	F 99%
Treatment	5	11,285	2,257	7.10**	2.55	3.73
Error	29	9,178	316			
Total	34	20,463				

** Significant at the 1% level of probability

Measurement 4

Source	DF	SS	MS	F cal	F 95%	F 99%
Treatment	5	4,403	881	1.40	2.55	3.73
Error	29	18,245	629			
Total	34	22,648				

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Figure 35

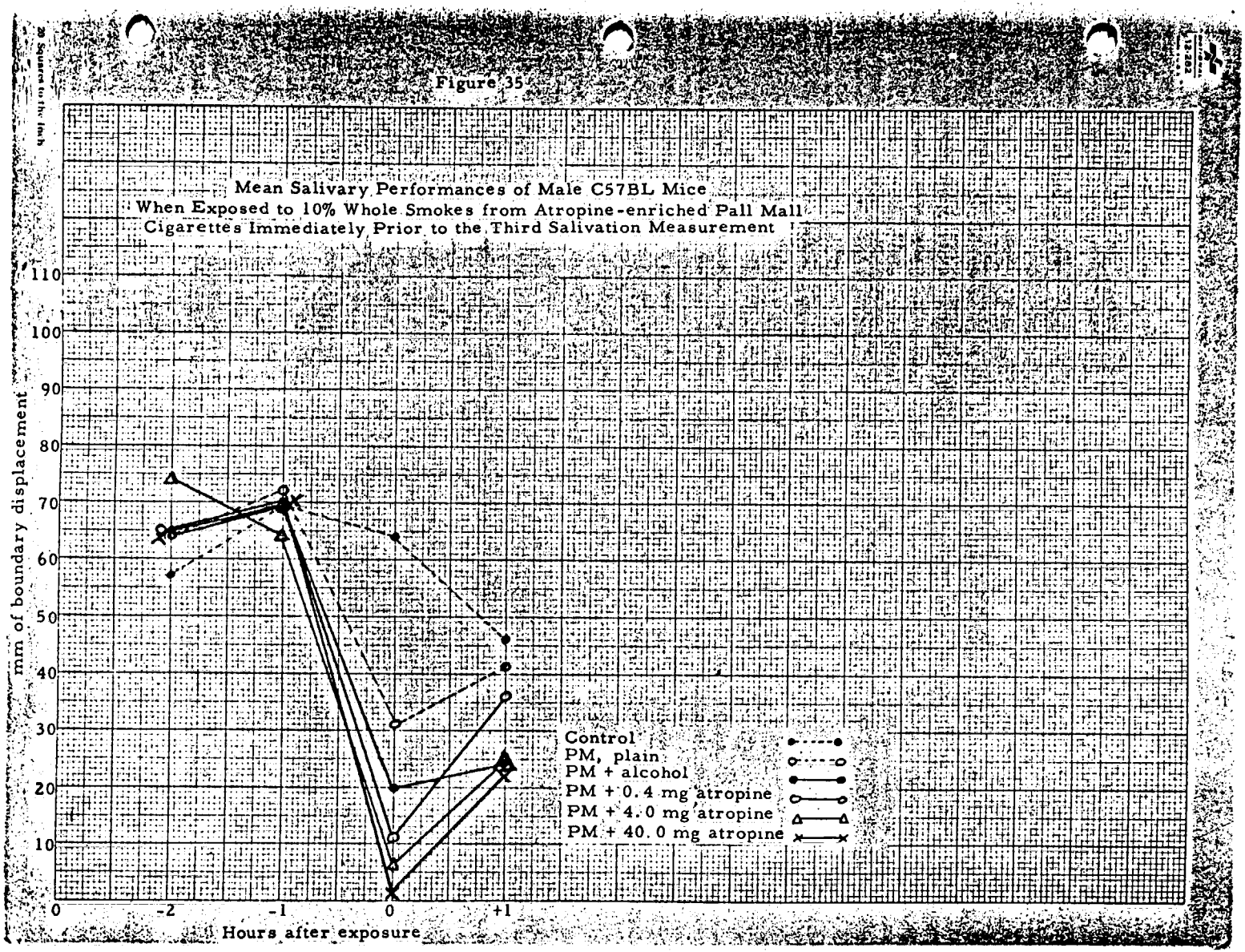

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Figure 36

Mean Salivary Performances of High-salivating Male C57BL Mice When Exposed to 10% Whole Smokes from Atropine-enriched Pall Mall Cigarettes Immediately Prior to the Third Salivation Measurement

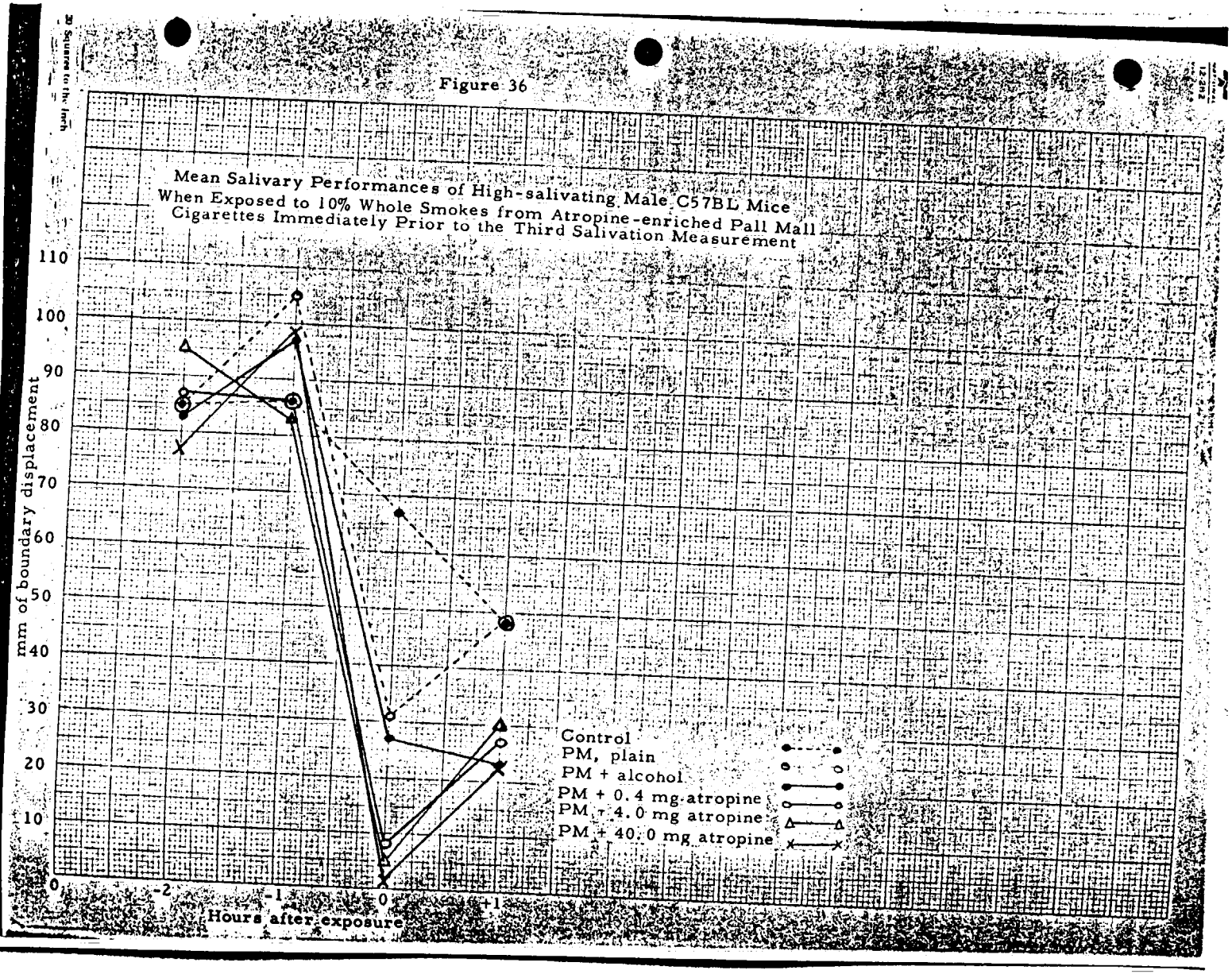

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Figure 37

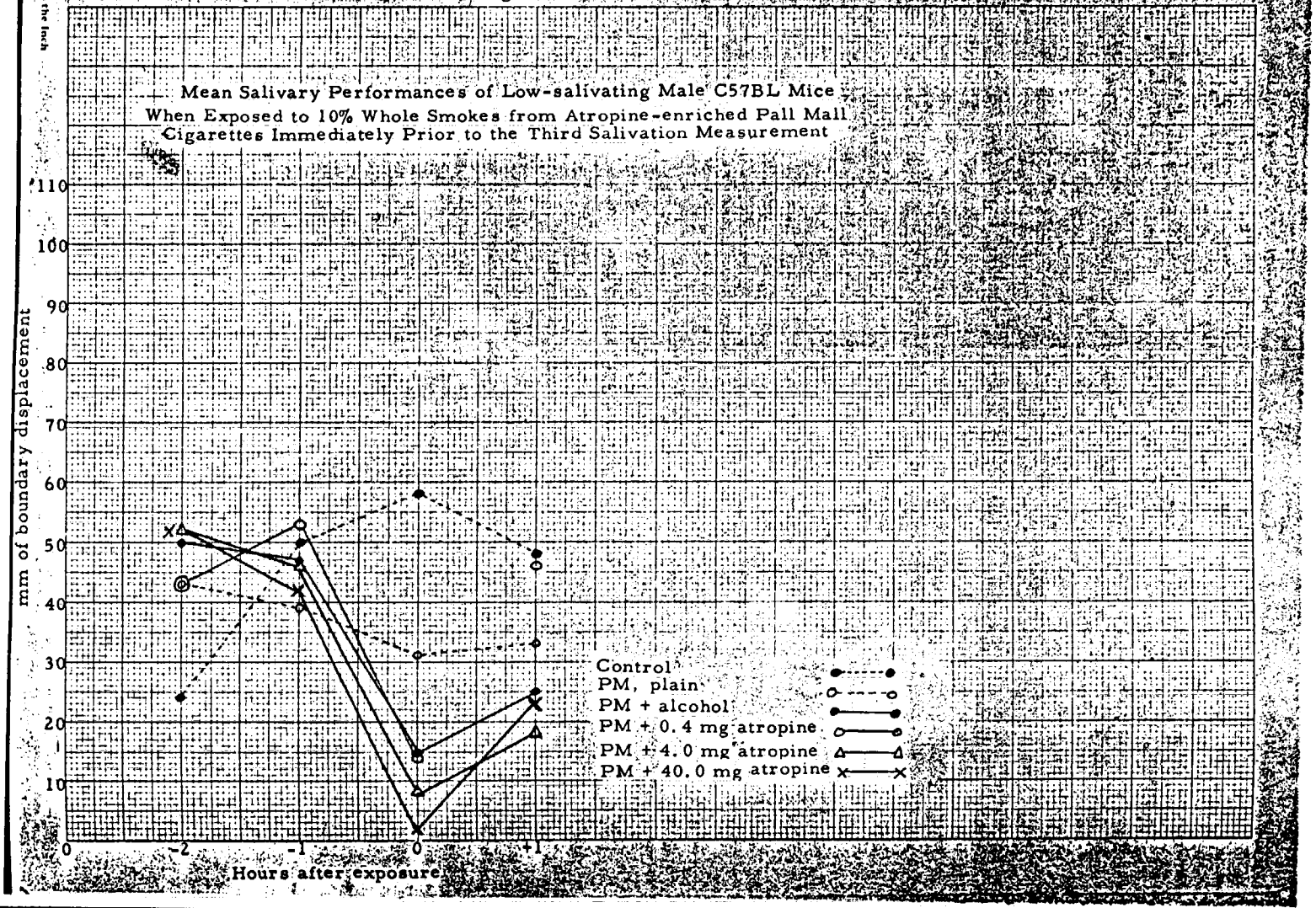

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Figure 38

EFFECT OF WHOLE SMOKES FROM PALL MALL (PM) CIGARETTES ENRICHED WITH VARYING DOSES OF ATROPINE (AT) ON SALIVARY PERFORMANCE OF POOLED, HIGH- AND LOW-SALIVATING MALE C57BL/6J MICE (MEASURED IMMEDIATELY AFTER EXPOSURE)

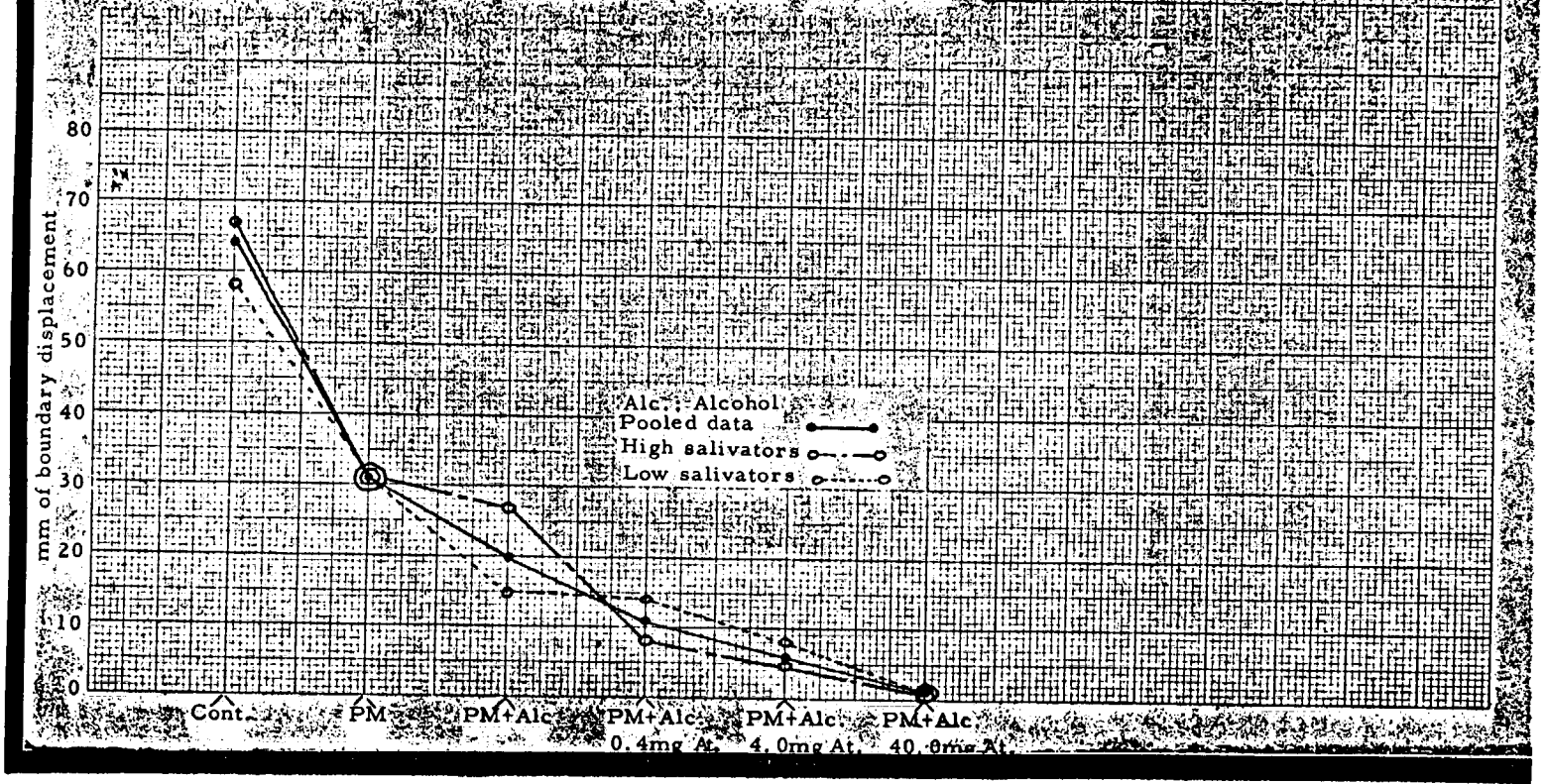

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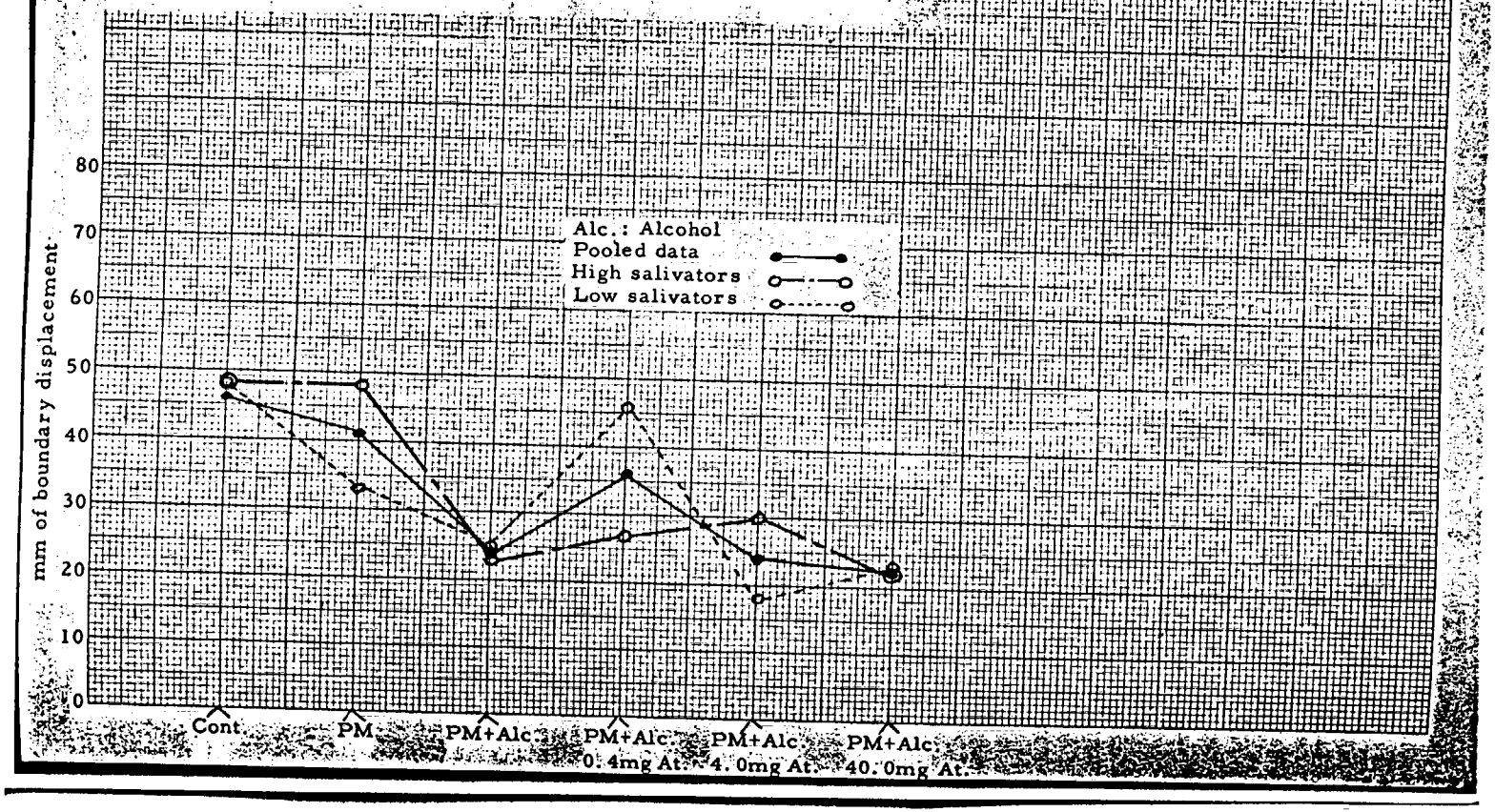
Figure 39

EFFECT OF WHOLE SMOKES FROM PALL MALL (PM) CIGARETTES ENRICHED WITH VARYING DOSES OF ATROPINE (AT) ON SALIVARY PERFORMANCE OF POOLED, HIGH- AND LOW-SALIVATING MALE C57BL/6J MICE (MEASURED ONE HOUR AFTER EXPOSURE)



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A NEW METHOD FOR THE MEASUREMENT OF SALIVATION IN SMALL RODENTS*

by

C. G. Van Dongen and F. Homburger

Bio-Research Consultants, Inc.
Cambridge, Massachusetts

* Paper presented at the fall meeting of the American Society for Pharmacology and Experimental Therapeutics, August 24-28, 1969, University of Pittsburgh, Pittsburgh, Pa.

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A New Method for the Measurement of Salivation in Small Rodents*

C. G. Van Dongen and F. Homburger

Bio-Research Institute, Cambridge, Mass. 02141

Mr. Chairman, members of the Society:

Studies on the salivary glands and their secretions are important with respect to nutrition, digestion, and dentistry, as well as with regard to excretion from circulation, and the neural and endocrinological regulation of glandular function.

The literature does provide a variety of methods to measure salivation. These have most frequently been worked out for the larger farm animals, as well as larger laboratory animals, such as dogs, cats, rabbits and rats. There are few methods available for smaller experimental animals, in particular, for hamsters or mice. This is due primarily to difficulties in the collection of saliva from these small species. Quite recently, however, some methods have been proposed for the measurement of salivary performance in mice. These procedures require anesthetized animals, and stimulation of salivation by drugs is essential in order to obtain information on the inhibition of salivation by various factors. Moreover, their monitoring system is not sensitive enough to quantitate salivation in the pseudo-physiological range (Richter; Lavy and Mulder; Aust).

To the best of our knowledge, the literature does not provide a simple method for the measurement of salivation in unanesthetized small rodents under pseudo-physiological conditions.

It is the purpose of this paper to present a nontraumatic method for the quantative monitoring of salivation in minimally restraint unanesthetized mice. Narrow cotton fabric strips pretreated with Cresol Red indicator are inserted into the mouth by means of an oral wire bit to absorb saliva. The length of the diffused colored band, visible after a given time interval, is measured in mm, and indicates the amount of saliva excreted. Immobilization of the mice for measuring salivation is achieved by fitting permanent "halters" around the neck of the animals and inserting the halter into a "stancion" during measurements. Such an arrangement permits the simultaneous salivation study of a large "herd" of mice.

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* Paper to be presented at the fall meeting of the American Society for Pharmacology and experimental therapeutics; August 24-28, 1969; Univ. of Pittsburgh, Pittsburgh, Pa.

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Slide I shows a closeup of a mouse in the stancion. The mouse wears a felt collar permanently around its neck. A number is engraved into the collar for identification of the mouse. During the experiment, the collar is fixed into this plexiglass holder. A wire "bit" is placed into the mouse's mouth. A narrow cotton strip is attached to the "bit." The strip turns from yellow to purple upon exposure with saliva.

Slide II shows, that the described setup does permit the simultaneous study of large numbers of mice. It demonstrates further that application of treatments can be done in a very systematic fashion. On this picture you see the salivary performance by various strains of mice (on which we will report later).

In order to standardize the method, spontaneous salivation was measured under a variety of experimental conditions over a ten-minute period. Salivation was found to be significantly affected, among other factors, by the number of consecutive days during which each mouse was subjected to measurement, the frequency of measurements during each day, and variations from one animal to another.

To further standardize the methodology, the method was applied to examine the presence or absence of any systematic differences among both sexes of various strains of mice. Twenty animals (half males, half females) of each of four inbred strains of mice, namely AKR/J, DBA/2J, C3HeB/FeJ and C57BL/6J from the Jackson Laboratories in Bar Harbor were studied. Salivation was measured over a four-day period, six times daily at hourly intervals.

Slide III presents the design of this experiment, involving a total of 80 mice and 1,920 individual salivation determinations.

The mice, all ten weeks old, were housed five to a cage for a period of two weeks prior to the actual start of the salivation experiment. During the first week, all mice were fitted with collars and numbered. The mice had no prior exposure to the salivation technique. The data were systematically recorded. They were analyzed statistically, primarily by means of the analysis of variance, using the 370 Wang computer system.

We will limit ourselves to statistically significant results only, and exclude nonsignificant data or trends from further considerations.

Slide IV shows graphically the salivating performance for the four different strains of mice on four consecutive days. Significant differences among these strains were found, except between DBA/2J and the C57BL/6J. Thus, the AKR/J exhibits the lower salivation, salivary performance of the C3HeB/FeJ is intermediate, and DBA/2J and C57BL/6J are both high salivators.

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Slide V shows the salivary performances of mice in each sex of each strain. Statistical analysis of the data proved that male DBA/2J mice produced less saliva than the females of this strain. This sex difference was reversed in the C3HeB/FeJ, where the males produced more saliva than the females. No sex differences were noted in the low-salivating AKR/J and the high-salivating C57BL/6J mice.

Note further that salivation is significantly lower during the first day than in the three following days. This holds true for each sex of each of the four strains. The differences in salivation during the last three days are not significant, except for the AKR/J females, where salivation is increased progressively during the four-day period of experimentation.

We have done further statistical analysis on these data.

Slide VI provides a summary of the various factors that contribute to salivation differences in each sex and strain during this experiment.

The results show that in nearly all cases, more than 95 per cent of the variability in salivation can be accounted for by controlled factors, thus leaving only a 5 per cent random variability. This indicates that the experimental conditions within the inbred strains of mice are very well defined. Approximately 80 per cent of the variability in salivation can be attributed to individuality among the animals and to days of experimentation. The rank order of the measurement during the day, and the "Day X measurement" interaction, are only minor contributing factors to variability and were found to be nonsignificant. This means, in practice, that the spontaneous salivation during any one day is rather stable, when salivation is measured at hourly intervals.

In future biological assays, we will utilize the contributions to variability by "animals" but will eliminate the contribution to variability by "days" through omission of first day salivation data from mice not previously used.

As an example in pharmacology, a pilot study was performed examining the effect of methacholine on salivary performance of random-bred male Ha/ICR Millerton Swiss mice, (a noninbred strain which exhibits much more variability in salivating performance than the inbred strains of mice, presented above).

Slide VII shows the results obtained. Note the large stimulation of salivation when the mice were injected subcutaneously with methacholine, immediately prior to the third salivation.

In summary, a new method has been described for the measurement of salivation in small rodents. The method employs minimally restraint, unanesthetized mice. The method is suitable for physiological and pharmacological studies, and data can be obtained simply and in adequate numbers to permit statistical analysis of results.

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EXPERIMENTAL DESIGN (STRAIN STUDY)

STRAIN	SEX	NO. ANIMALS	DAYS 1	2	3	4
AKR/J	M	10				
	F	10				
DBA/2J	M	10				
	F	10				
C3HeB/FeJ	M	10				
	F	10				
C57BL/6J	M	10				
	F	10				

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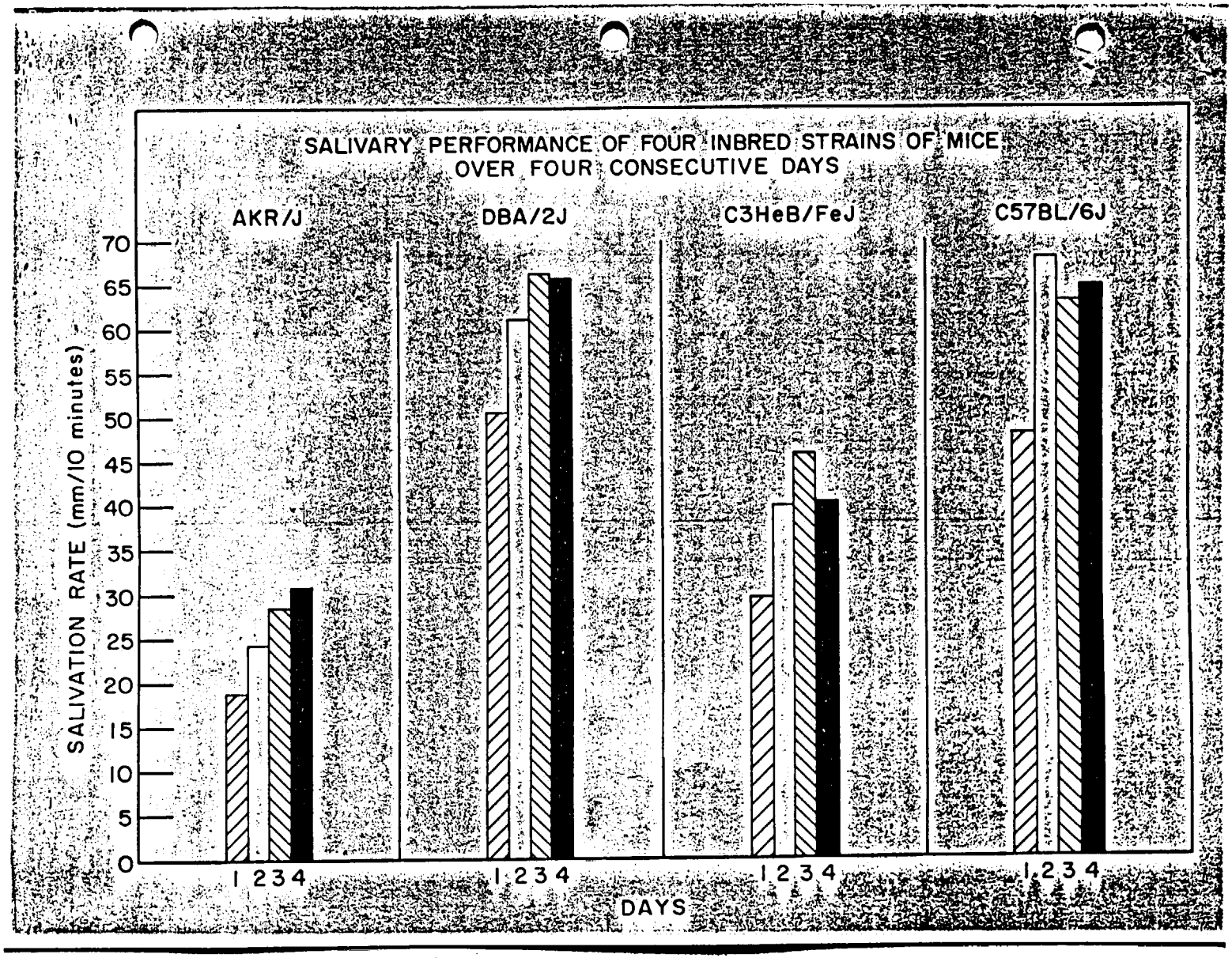

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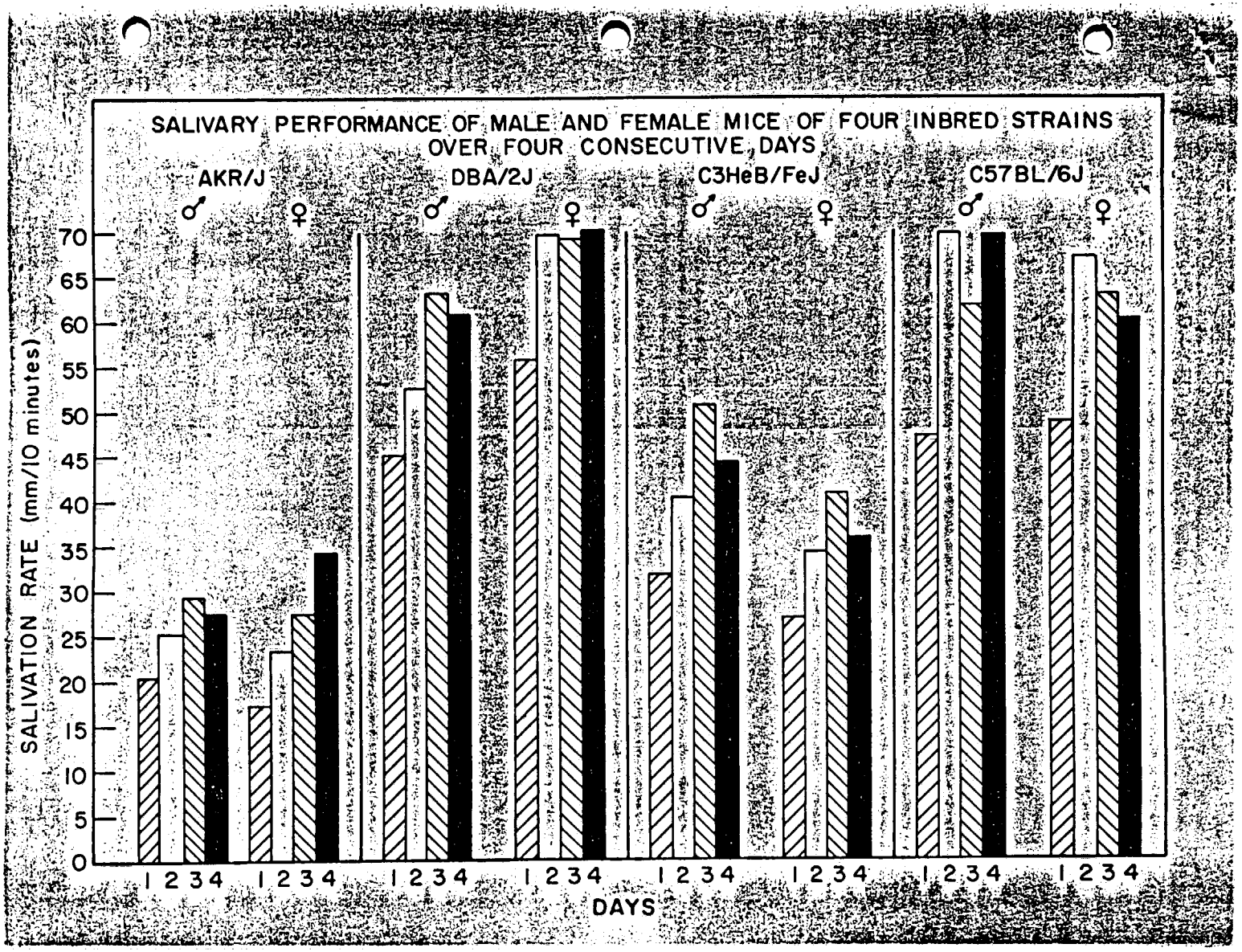

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Summary of Factors Contributing to Total Variance in Salivary Performance in Each Sex of Four Inbred Strains of Mice (Expressed in Percentages)

Strain	AKR/J (I)		DBA/2J (II)		C3HeB/FeJ (III)		C57BL/6J (IV)	
Sex	M	F	M	F	M	F	M	F
Animals	59**	30**	42**	58**	40**	33**	33**	34**
Times	6*	9**	8**	6	8**	9	11**	12**
Days (D)	21*	56**	39**	27**	38**	46**	40**	31**
Measurements (M)	7	1	5	3	7	2	7	9**
D x M	3	2	3	3	3	4	6	10**
Error	4	2	4	4	4	7	3	4
Total	100	100	100	100	100	100	100	100

** Significant at the 1% level of probability.
* Significant at the 5% level of probability.

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Source: https://www.industrydocuments.ucsf.edu/docs/lklk0000

THE EFFECT OF METHACHOLINE Cl ADMINISTERED SUBCUTANEOUSLY IMMEDIATELY PRIOR TO THE THIRD MEASUREMENT ON SALIVARY PERFORMANCE OF MALE Ha/ICR MILLERTON SWISS MICE

TREATMENT	MEASUREMENT			
	1	2	3	4
CONTROL	183*	127	164	159
0.083 MG	141	188	373	143
0.833 MG	157	103	812	97
8.333 MG	154	196	DIED	---

* MEAN OF TEN SALIVATION MEASUREMENTS, EXPRESSED IN MM.

100311289

Source: https://www.industrydocuments.ucsf.edu/docs/lklk0000

BIOASSAY FOR MEASUREMENT OF CHOLINERGIC AND ANTICHOLINERGIC DRUG POTENCIES IN MICE*

by

C. G. Van Dongen and F. Homburger

Bio-Research Consultants, Inc.
Cambridge, Massachusetts

* Paper presented at the annual meeting of the American Society for Pharmacology and Experimental Therapeutics, April 12-17, 1970, Atlantic City, N.J.

100312893

Source: https://www.industrydocuments.ucsf.edu/docs/lklk0000

BIOASSAY FOR MEASUREMENT OF CHOLINERGIC AND ANTICHOLINERGIC DRUG POTENCIES IN MICE.*

C. G. Van Dongen and F. Homburger, Bio-Research Institute, Cambridge, Massachusetts 02141.

Mr. Chairman, members of the Society:

We reported previously on a nontraumatic method for the quantitative measurement of salivation in minimally restrained unanesthetized mice.

Narrow cotton fabric strips, pretreated with cresol red indicator, are inserted into the mouth by means of an oral wire bit to absorb saliva. The length of the diffused colored band, visible after a given time interval, is measured in mm, and indicates the amount of saliva excreted. Immobilization of the mice for measuring salivation is achieved by fitting "collar" around the neck of the animals and inserting the collar into a "stanchion" during measurements. Such an arrangement permits the simultaneous study of salivation in a large "herd" of mice.

Slide I shows a mouse in a stanchion by its permanently worn collar. The mouse bites on a wire "bit." A narrow cotton strip, attached to the bit turns from yellow to purple upon exposure to saliva.

Next slide shows that a large number of mice can be studied simultaneously. Significant differences in salivary performance exist among various strains of mice.

High salivating C57BL mice, low salivating AKR/J mice, all supplied by the Jackson Laboratories in Bar Harbor, Maine.

It is the purpose of this paper, first to describe the immediate and aftereffects of two drugs on salivary performance of AKR and C57BL mice (the two drugs are methacholine chloride and atropine sulfate), second, to examine the feasibility and to determine the optimal conditions of this experimental approach as a bioassay for the measurement of cholinergic and anticholinergic drug potencies.

The next slide presents the experimental layout to study the effect of methacholine on salivation. A similar experimental design was followed for atropine. Male AKR and C57BL mice were used. Salivation of the

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* Paper to be presented at the annual meeting of the American Society for Pharmacology and Experimental Therapeutics, April 12-17, 1970 in Atlantic City, New Jersey.

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Source: https://www.industrydocuments.ucsf.edu/docs/lklk0000

mice was measured eight times during one day at intervals of one hour. Measurements 1 and 2 served as controls to establish the salivary performance of individual mice. These salivary performances then served as a basis for randomization of the mice into one of nine treatment groups. Immediately prior to the third measurement, drug dosages were applied. Thus the third salivation measurement indicates the immediate drug effect. Measurements 4 through 8 were included to quantitate any aftereffects to the drug.

The nine treatments applied were "control," "water" and a logarithmic series of doses of methacholine, ranging from 0.1 through 100 μg, injected subcutaneously in 0.1 ml of water.

It is of interest to point out that any drug treatment can thus validly be compared with two controls, namely first with the "control" and "water" treatment groups, but second also with their own salivation performances during the first and second measurements, as earlier results had shown that no significant differences in salivation exist during the day.

Part of the data will now be presented to illustrate the overall salivation pattern in two strains of mice, caused by methacholine or atropine.

The next slide shows the effect of 0.3, 3.0 and 30 μg methacholine on the salivary performance of high salivating C57BL mice. Note that methacholine stimulates salivation initially and mice over a relative small dose range (from a control level of 60 to 130 mm for 30 μg of methacholine). The salivary stimulation is afterwards changed to a salivary inhibition.

The next slide shows the same treatments of methacholine on salivation of low-salivating AKR mice. Note that the salivary increase is much more pronounced (from a control level of 15 to 160 mm with 30 μg of methacholine).

The next slide shows the effect of atropine on salivary performance of low-salivating AKR's. There is a relative small salivary decrease (from control level of 30 to 5 mm with 30 μg of atropine).

The next slide shows the salivary inhibition by atropine in high-salivating C57BL mice. Obviously, this inhibition from a control level of 47 to 3 mm with 30 μg of atropine, is much more dramatic than that shown in the previous slide with AKR mice.

The foregoing results suggest that it is feasible to use the salivary stimulation of a low-salivating strain, and the salivary inhibition of a high-salivating strain of mice for bioassay purposes.

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We will now examine in some detail the immediate responses of methacholine on salivation of AKR's and of atropine on the salivation of C57BL mice.

The next slide shows the relationship between a series of doses of methacholine and immediate salivation in male AKR mice. The experimental animals were separated into high and low salivating individuals within the AKR strain. A good dose-response relationship is noticeable with each group over the dose range of 0. through 30 μg of methacholine.

The next slide shows the calculated linear regressions and indices of precision over the 0.1-30 μg dose range. The indices of precision are 0.79 and 0.71, respectively, indicating a slight improvement of the method through application of preselection of individuals within the AKR strain.

The next slide summarizes the relative usefulness of high and low-salivating C57BL, and the high and low-salivating AKR/J for bioassay purposes of methacholine. The threshold of methacholine is below 0.1 μg for each, but the linear dose range, as well as the indices of precision show that low-salivating AKR's are superior to any other mice.

The next slide shows the relationship between a series of doses of atropine and immediate salivation inhibition of male C57BL mice. Here again, the experimental animals were separated into high or low-salivating individuals within the C57BL strain. A good dose-response relationship can be seen over the range 0.3 through 30 μg of atropine.

The next slide shows the calculated linear regressions over the same dose range. Although the slopes are smaller than those of the D - R curves with methacholine, the variability is also smaller. This is indicated by the favorable ' of 0.45 and 0.53, respectively.

The next slide summarizes the relative usefulness of high and low-salivating C57BL and high and low-salivating AKR's for bioassay purposes of atropine. The best index of precision was indeed obtained with preselected high-salivating C57BL mice.

In summary

A new experimental approach has been presented for the measurement of salivation in rodents. The method employs minimally restraint unanesthetized mice, and is suitable for pharmacological studies on salivation.

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The experimental design can be arranged to study both the immediate and aftereffects effectively, and data can be obtained simply and in adequate numbers to permit statistical analysis of results.

Methacholine was found to stimulate salivation initially, followed by a salivary inhibition. Atropine, on the other hand, only showed salivary inhibition. These effects are dose-dependent.

The immediate effects of methacholine and of atropine were explored for bioassay purposes. Optimal conditions for the bioassay of methacholine require the use of low-salivating individuals from a low-salivating strain of mice. For the bioassay of atropine, one should use high-salivators from a high-salivating strain of mice.

The threshold dose of each drug is smaller than 0.1 μg; the linear dose range is at least one hundred; and the indices of precision are acceptable.

In conclusion, this new approach which uses the salivation of mice, is suitable for bioassay purposes of cholinergic and anticholinergic substances.

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Experimental Design (Bioassay Cholinergics)

Mouse Strain	Dose (μg)	No. of Animals	Measurement							
			1	2	3	4	5	6	7	8
AKR/J	Control	12								
	0.0	12								
	0.1	12								
	0.3	12								
	1.0	12								
	3.0	12								
	10.0	12								
	30.0	12								
	100.0	12								
C57BL/6J	Control	12								
	0.0	12								
	0.1	12								
	0.3	12								
	1.0	12								
	3.0	12								
	10.0	12								
	30.0	12								
	100.0	12								

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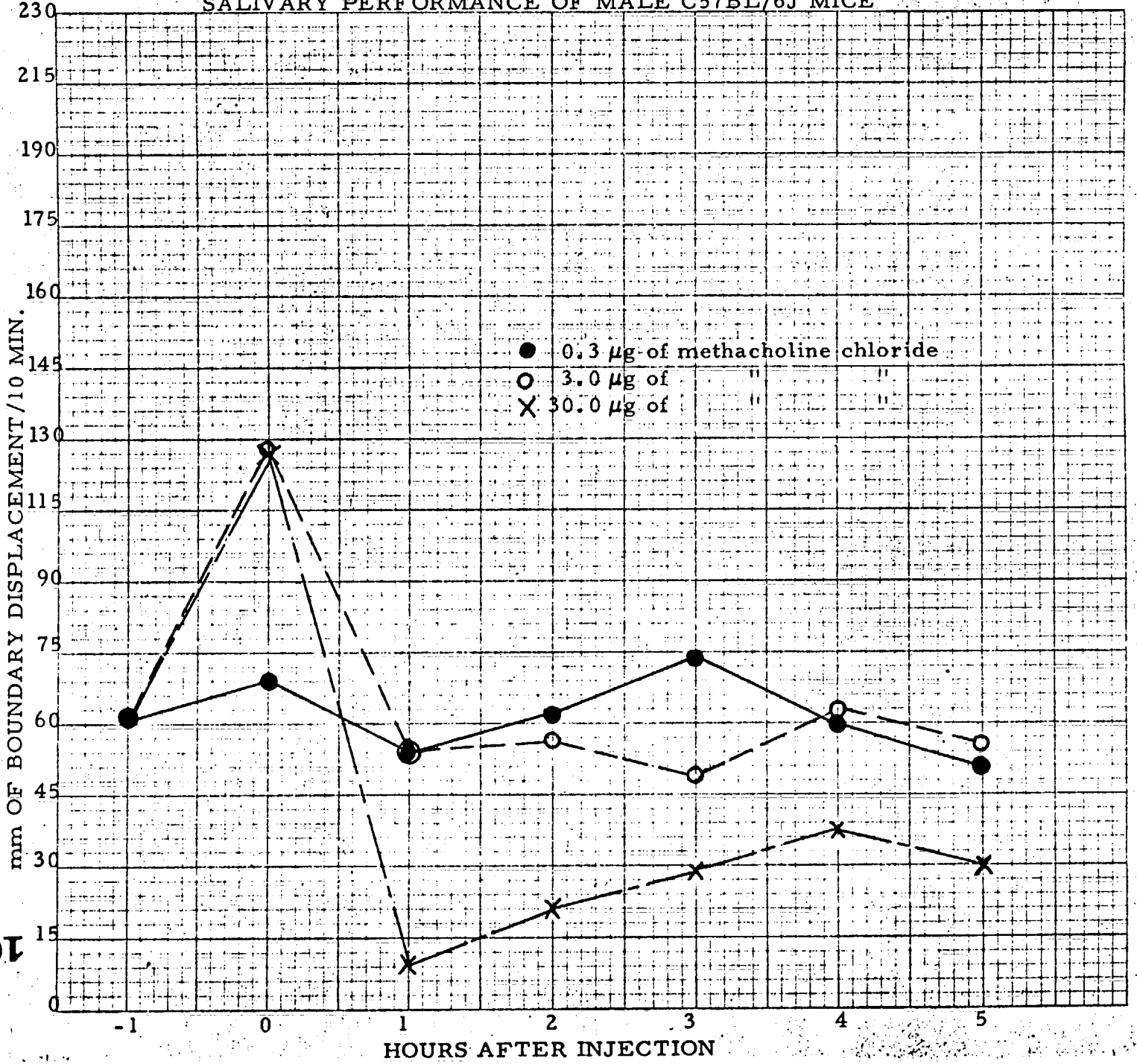
Source: https://www.industrydocuments.ucsf.edu/docs/lklk0000

THE EFFECT OF VARIOUS DOSES OF METHACHOLINE CHLORIDE ON SALIVARY PERFORMANCE OF MALE C57BL/6J MICE



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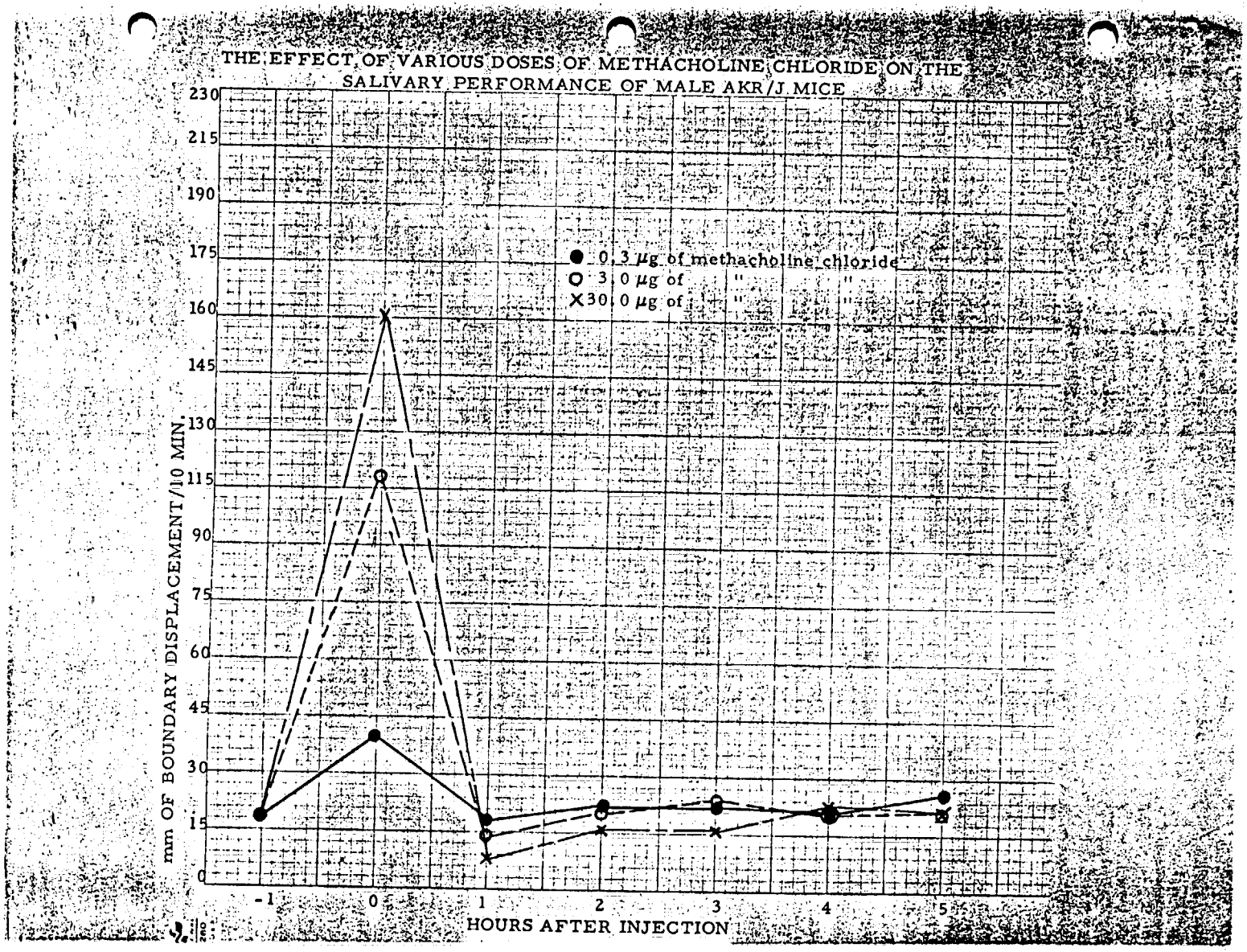
Source: https://www.industrydocuments.ucsf.edu/docs/lklk0000



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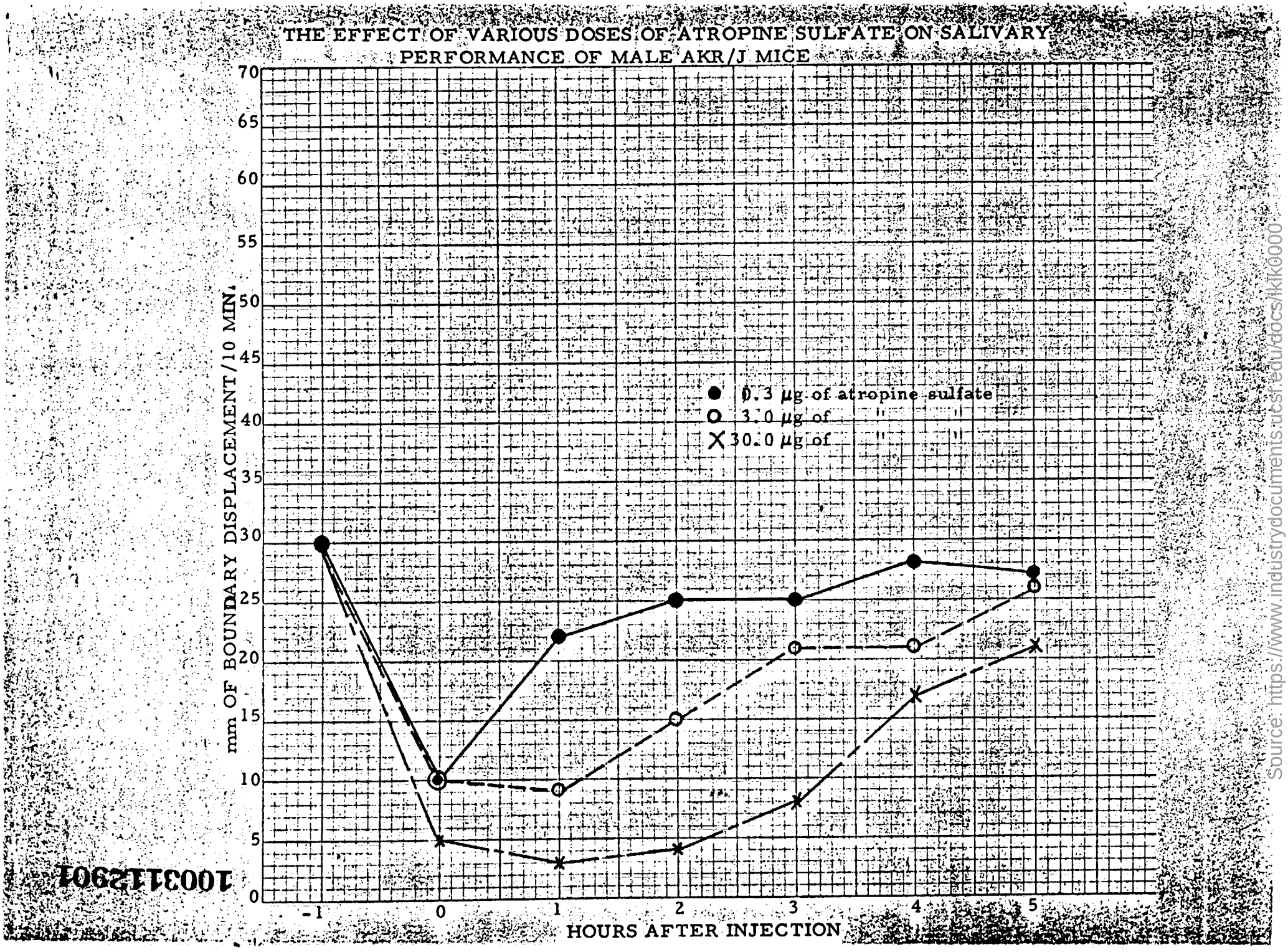
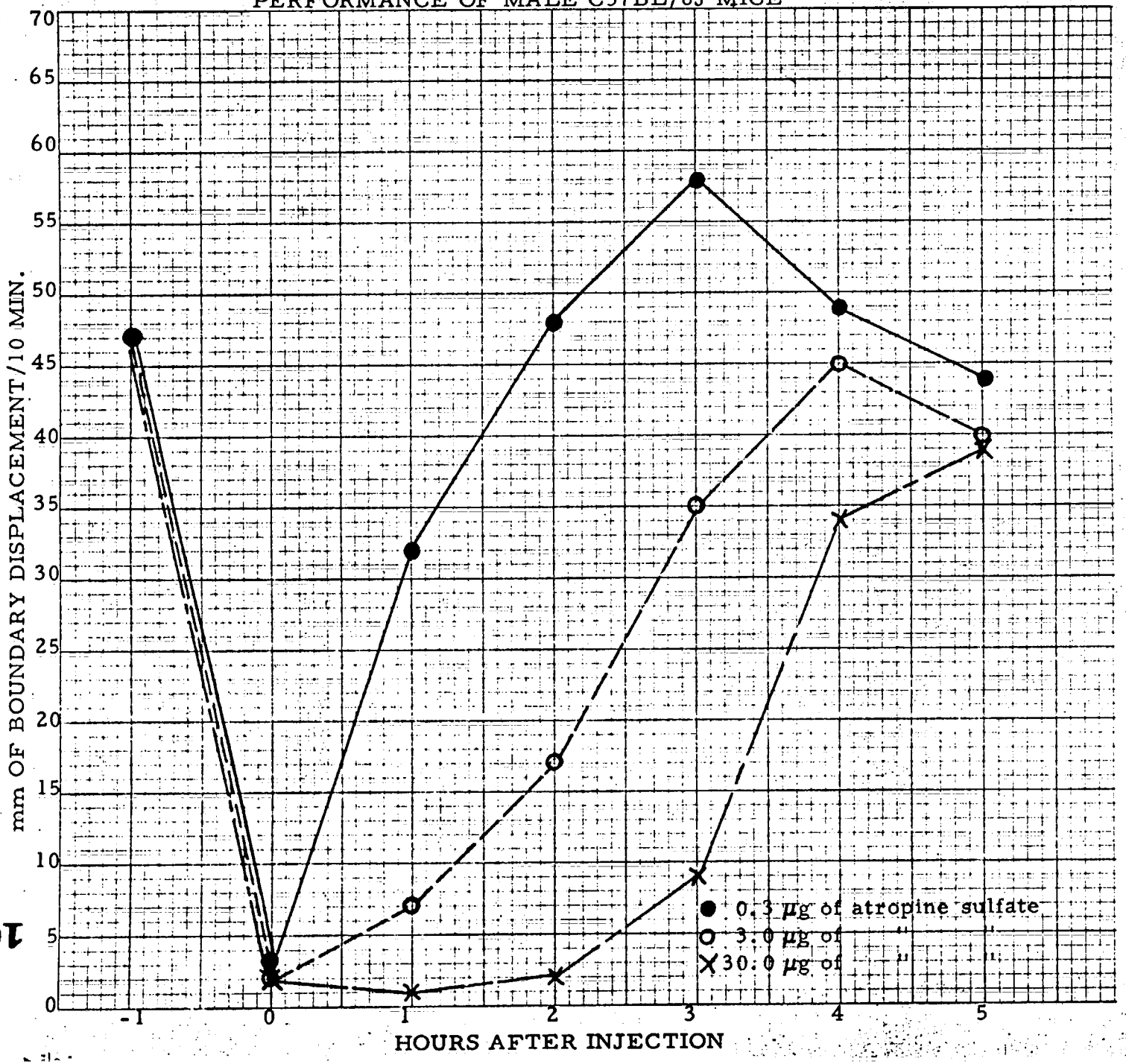
Source: https://www.industrydocuments.ucsf.edu/docs/lklk0000



Source: https://www.industrydocuments.ucsf.edu/docs/kkk0000

THE EFFECT OF VARIOUS DOSES OF ATROPINE SULFATE ON SALIVARY PERFORMANCE OF MALE C57BL/6J MICE



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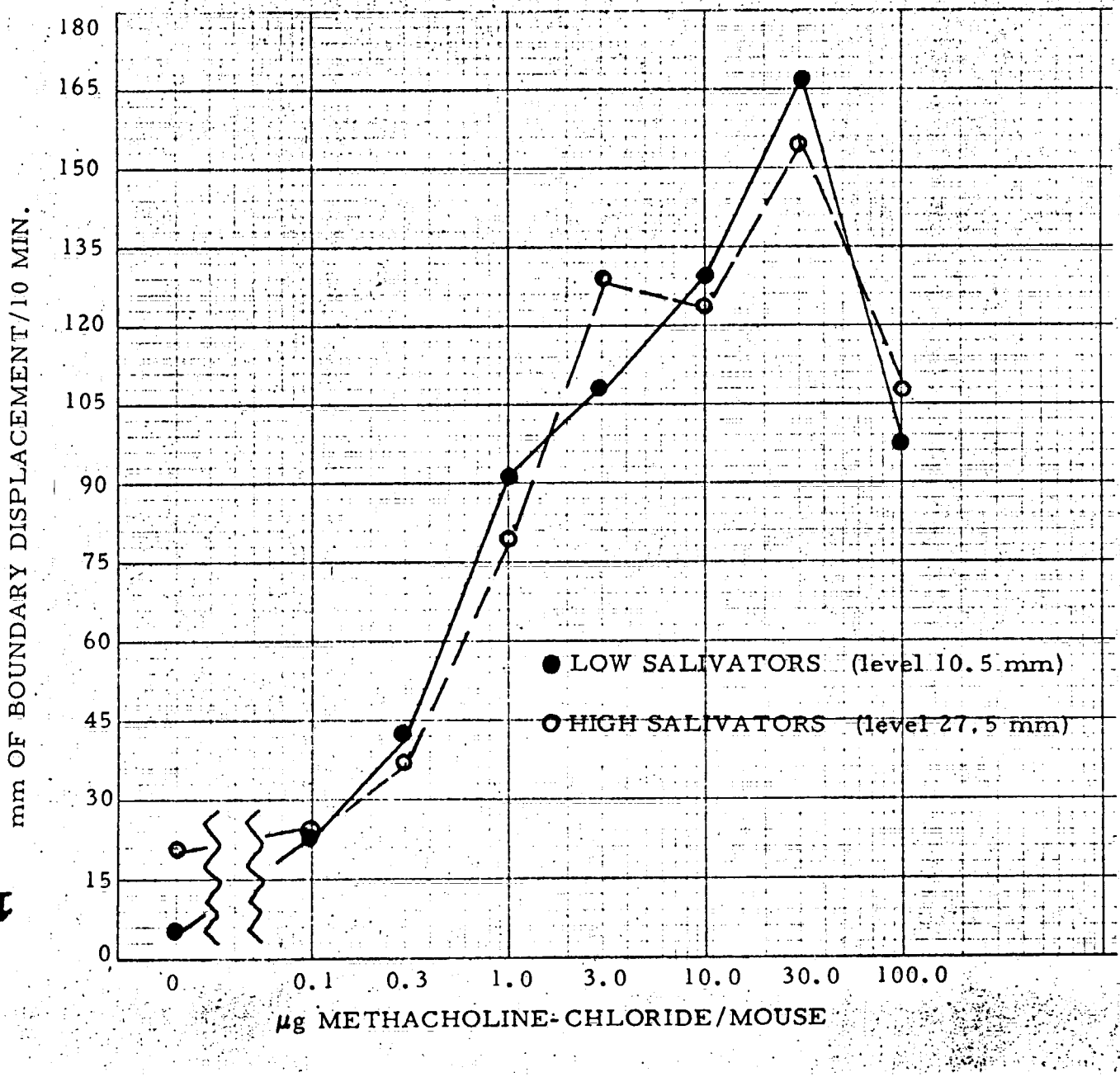
Source: https://www.industrydocuments.ucsf.edu/docs/lklk0000

RELATIONSHIP BETWEEN A SERIES OF DOSES OF METHACHOLINE CHLORIDE AND IMMEDIATE SALIVATION IN MALE AKR/J MICE PRESELECTED FOR HIGH AND LOW SALIVARY PERFORMANCE



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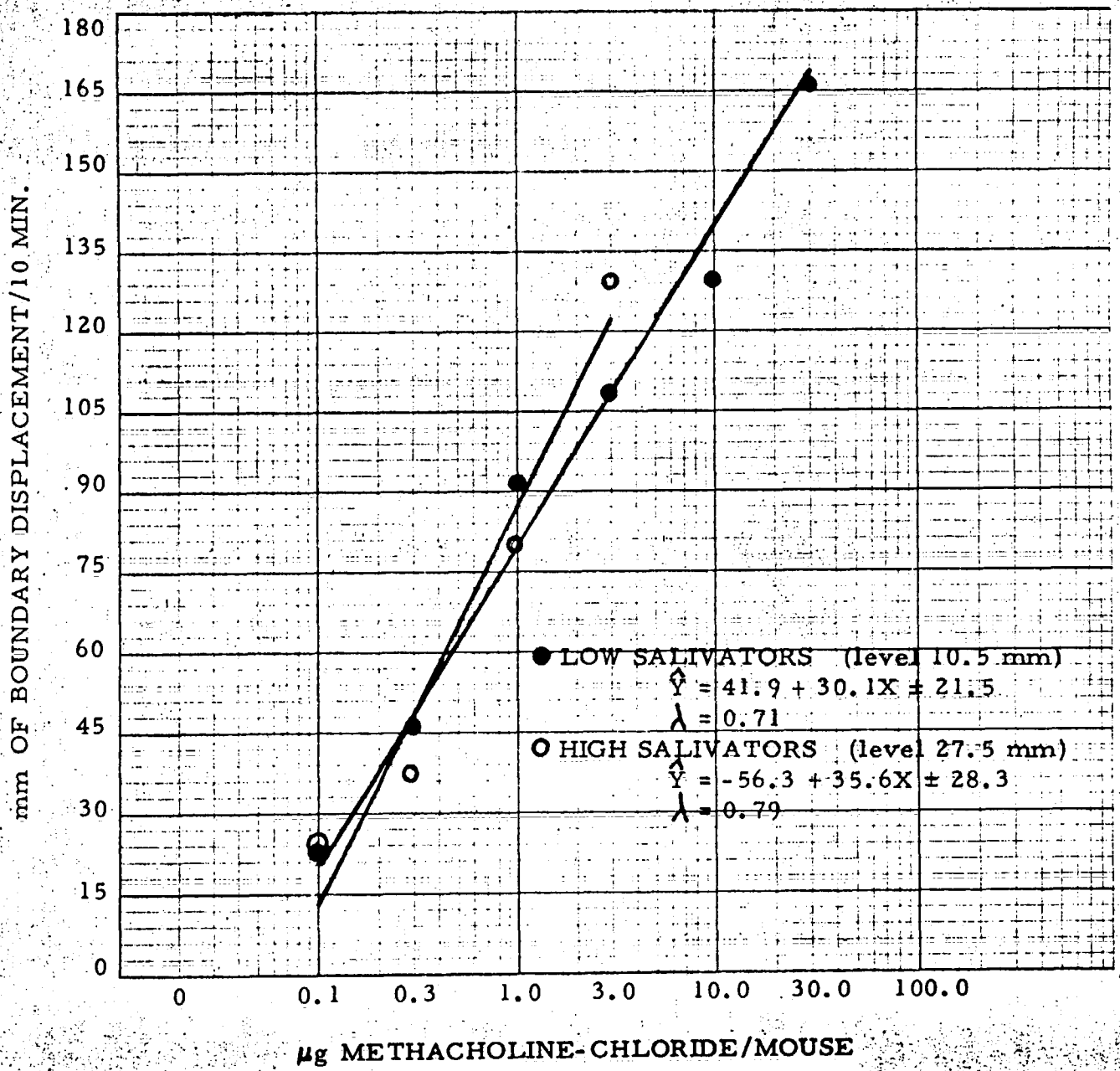
Source: https://www.industrydocuments.ucsf.edu/docs/lklk0000

RELATIONSHIP BETWEEN A SERIES OF DOSES OF METHACHOLINE CHLORIDE AND IMMEDIATE SALIVATION IN MALE AKR/J MICE PRESELECTED FOR HIGH AND LOW SALIVARY PERFORMANCE (LINEAR PORTION ONLY)



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Source: https://www.industrydocuments.ucsf.edu/docs/lklk0000

Characteristics of the Dose-Response Relationship between a Logarithmic Series of Doses of Methacholine Chloride and the Immediate Salivation in Two Strains of Male Mice with Varying Salivary Performance (Linear Portion Only)

	Threshold (μg)	Linear Range (μg)	r	Regression $\hat{Y} = a + bX \pm S_{yx}$	λ
C57BL/6J					
high	< 0.1	0.3- 3.0	0.43	31.5+18.8X±34.7	1.85
low	< 0.1	0.3- 3.0	0.74	-69.2+39.8X±31.0	0.78
AKR/J					
high	< 0.1	0.1- 3.0	0.84	-56.3+35.6X±28.3	0.79
low	< 0.1	0.1-30.0	0.91	-41.9+30.1X±21.5	0.71

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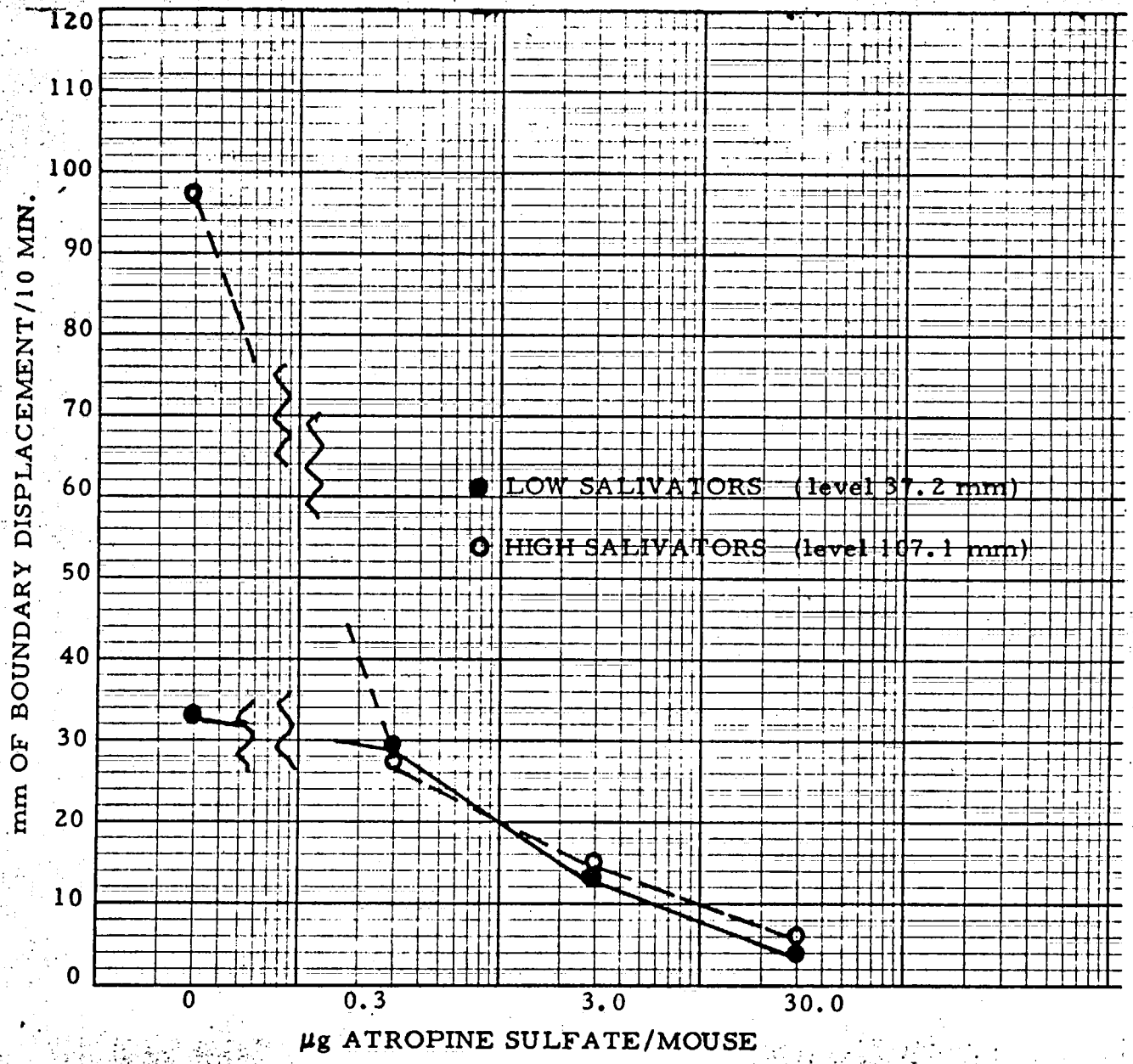
Source: https://www.industrydocuments.ucsf.edu/docs/lklk0000

RELATIONSHIP BETWEEN A SERIES OF DOSES OF ATROPINE SULFATE AND IMMEDIATE SALIVATION INHIBITION OF MALE C57BL/6J MICE PRESELECTED FOR HIGH AND LOW SALIVARY PERFORMANCE



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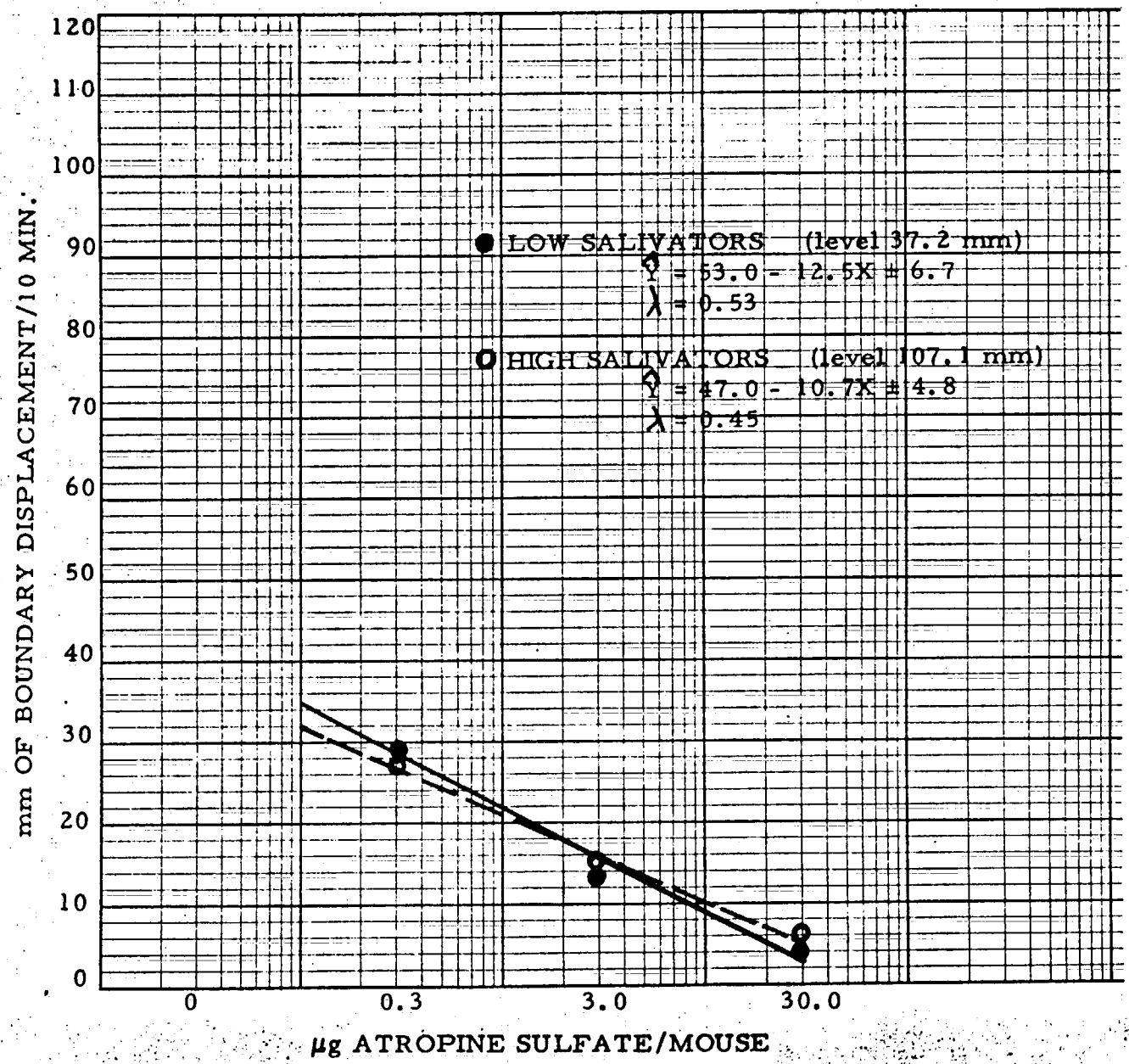
Source: https://www.industrydocuments.ucsf.edu/docs/lklk0000

LINEAR REGRESSION BETWEEN A SERIES OF DOSES OF ATROPINE SULFATE AND IMMEDIATE SALIVATION INHIBITION IN MALE C57BL/6J MICE PRESELECTED FOR HIGH AND LOW SALIVARY PERFORMANCE



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Source: https://www.industrydocuments.ucsf.edu/docs/lklk0000

Characteristics of the Dose-Response Relationship between a Logarithmic Series of Doses of Atropine Sulfate and the Immediate Salivary Inhibition in Two Strains of Male Mice Preselected for High and Low Salivary Performance

	Threshold (μg)	Linear Range (μg)	r	Regression $\hat{Y} = a + bX \pm S_{yx}$	λ
C57BL/6J					
high	< 0.1	0.3-30	0.89	47.0-10.7X±4.8	0.45
low	< 0.1	0.3-30	0.85	53.0-12.5X±6.7	0.53
AKR/J					
high	< 0.1	0.3-30	0.52	33.1- 6.4X±9.3	1.45
low	< 0.1	0.3-30	0.50	23.3- 3.9X±5.9	1.51

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Source: https://www.industrydocuments.ucsf.edu/docs/lklk0000

RELATIONSHIP IN MICE BETWEEN INHIBITION OF SALIVATION AND TOXICITY OF CIGARETTE SMOKE*

by

C. G. Van Dongen
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1003112909

* Paper to be presented at the annual meeting of the Society for Toxicology, to be held in Washington, D. C. on March 7-11, 1971.

Source: https://www.industrydocuments.ucsf.edu/docs/lklk0000

Mr. Chairman, Members of the Society:

We reported previously on a non-traumatic method for the quantitative measurement of salivation in minimally restrained unanesthetized mice.

Narrow cotton fabric strips, pretreated with cresol-red indicator, are inserted into the mouth by means of an oral wire bit to absorb saliva. The length of the diffused colored band, visible after a given time interval, is measured in mm. and indicates the amount of saliva excreted. Immobilization of the mice for measuring salivation is achieved by fitting a "collar" around the neck of the animals and inserting the collar into a "stanchion" during measurements. Such an arrangement permits the simultaneous study of salivation in a large "herd" of mice.

Slide I shows a mouse in a stanchion by its permanently worn collar. The mouse bites on a wire bit. A narrow cotton strip, attached to the bit turns then from yellow to purple upon exposure to saliva.

The next slide shows that a large number of mice can be studied simultaneously. Significant differences in salivary performance exist among various strains of mice. High-salivating C57BL mice. Low-salivating AKR mice. All supplied by the Jackson Laboratories in Bar Harbor, Maine.

The above described salivation method in mice has been applied previously to study the effects of various physiological factors, such as sex and strains on salivary performance. The method also proved to be a useful tool as a bio-assay for cholinergic and anti-cholinergic drug substances. Recent pilot experiments, moreover, showed that the exposure of mice to cigarette smoke reduced their salivation, and that this effect is less pronounced while only the gasphase of such smoke is employed. These findings suggest the possibility of a relationship between salivary performance and toxicity of cigarette smokes.

The specific objectives of this study are: (1) to establish the acute toxicities of whole smokes from these two brands of cigarettes; (2) to confirm or disprove the inhibitory action of whole tobacco smokes on salivary performance in a dose-related fashion; (3) to examine any differential inhibitory action between the whole smokes of two cigarette brands; (4) to examine some experimental conditions with the objective of obtaining the salivation inhibitory actions with higher resolution.

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To perform both types of experiments, a large herd of male C57BL mice was available. The animals were approximately 24 weeks old, had been housed for some time six to a cage with their collars on. They also had been adapted previously to the salivation procedure and were all in good health.

In the salivation experiment, salivary performance of mice was determined five times with a one-hour interval between measurements. The first and second measurements served to establish the control salivary performance of individual mice during that particular day. A ten-minute smoke exposure was applied immediately prior to the third measurement. The fourth and fifth measurements were performed to examine any after-effects to treatments. During the time intervals between any two measurements, the mice were returned to their cages which were supplied with adequate food and water. Salivary performance in mice was determined according to the above mentioned standard procedures.

Smoke exposures were applied with the Walton-reverse smoking machine. The conditions in the smoking machine were standardized to 35 ml. puffs of two seconds' duration taken at intervals of 58 seconds. The smoke would persist for 15 seconds in the inhalation chamber and then be expulsed in 3 seconds followed by a fresh-air purge for 40 seconds. Ten puffs were studied in each case, using the first 5 puffs of each of two sets of cigarettes, smoked without interruption in the one puff per minute cycle.

We applied any one of the following ten treatments, namely, no machine exposure, machine exposure with air, or any one of four smoke dilutions from whole smokes of brand A or B. The smoke dilutions were 1 to 5.4, 1 to 8.0, 1 to 16.0, and 1 to 32.0.

The cigarette brands A and B were selected on the basis of their tar and nicotine contents. Brand A had a high tar and nicotine content, whereas brand B had a low tar and nicotine content. The cigarettes were equalized in length to 65 mm. and climatized at 60% relative humidity in a descicator for at least several days until immediately prior to their actual use in the smoking machine.

The next slide shows the experimental layout of a single two-days' experiment on 72 mice. On the first day, the experiment started with determining a first and second salivation measurement on 36 mice. These mice were then rankordered according to salivary performance and systematically assigned to any one of six treatment groups. The various treatments, applied immediately prior to the third salivation, are thus

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done on very equal groups of mice. The second day's experiment on different mice was done the same, except for different smoke dilutions. Such a two-day experiment was repeated four times. Thus this study includes approximately 72 x 4 x 5 = 1440 salivation measurements.

The next slide presents a layout of the entire salivation experiment. It is noteworthy that any smoke exposures can be compared validly with two types of controls, namely, first with "no exposure" and "machine exposure only," but second also with their own salivary performances during the first and second measurements, as earlier results had shown that non-significant differences in salivation exist during the day. The two types of controls provide further an internal experimental tool to establish justification for pooling the data of single days into larger units for statistical analysis.

Statistical analysis of the data was done primarily using the analysis of variance technique.

In the acute toxicity experiments, groups of six mice were exposed to a 1 to 4.0 or a 1 to 8.0 dilution of whole smoke from either brand A or brand B. Such an experiment was repeated 4 times so that each treatment was applied to 24 mice. The exposures were continued until the mice died, with a maximal exposure time of 60 minutes. Toxicity was determined in terms of percentage of animals dead and by exposure time till death.

The next slide presents data on acute toxicity. The results show 100% lethality with the 1 to 4.0 dilution of whole smokes in each brand. Differential toxicity between brands was, however, found on the basis of exposure time. Death occurred faster with brand A than with brand B, 9.6 vs. 14.5 minutes.

The next slide shows that this effect is highly significant statistically. An F-value of 22.6, whereas only 4.1 is necessary at the 5% level of probability. No deaths occurred with the 1 to 8.0 dilution of either brand. The 60 minute cut-off might have prevented us from obtaining substantiating experimental evidence.

We can, however, conclude that acute toxicity of tobacco smoke depends, as expected, on smoke dilution and brand, and that brand A is more toxic than brand B.

Proceeding now with the results on salivary inhibition, one needs first to establish the validity of pooling the various single day's data before one can draw any valid conclusions from the entire experiment. This can be done in retrospect through comparing the base

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salivation levels of the mice already used for the various treatment groups. The second salivation measurement could well serve this purpose. In statistical terms, the variance due to "brand," "concentration," and the "brand x concentration" interaction should for the second measurement be negligible.

The next slide shows an analysis of variance on the salivary data from the second measurement. Note the very small calculated F-values in "brand," "concentration," and "b x c interaction." This indicates that the experimental animals were very well assigned on the basis of salivation level among the various treatments, and that treatment comparisons from the entire experiment can now validly be made.

The next slide shows the salivary performance of mice upon exposure to various dilutions of whole smoke from brand A. The abcisca represents the time from exposure, in hours, and the ordinate the level of salivary performance, expressed in mm. of boundary displacement. Note the approximate constancy over time and the non-significant differences between the two controls. These curves represent the salivary performances with the 1 to 32, the 1 to 16, the 1 to 8.0, and the 1 to 5.4 dilutions of whole smokes from brand A. Obviously a very dramatic salivary inhibition which is significantly more pronounced in size as well as duration, with higher smoke concentrations.

The next slide shows the salivary performance of mice upon exposure to whole smokes of brand B. It shows the same pattern as we saw with brand A.

The next slide shows the salivary inhibitions of brands A and B at each of the four smoke dilutions. Whereas any differential salivation inhibitory action between brands A and B is not noticeable at the 1 to 32 and 1 to 16 dilutions, a clear distinction can be made with the 1 to 8 and 1 to 5.4 dilutions. This shows that a minimal smoke concentration is required in order to start differentiating the two brands on the basis of salivary inhibition.

Having established the phenomenon that whole smokes exert in inhibitory effect on the salivation in mice, and having found that this salivary inhibition depends on brand and smoke concentration, it becomes then of interest to make optimal the experimental conditions to show this inhibitory effect. Thus we posed the questions: "Do any salivation inhibitory effects due to "brand" and "concentration" show up more pronounced immediately after or one hour after smoke exposure?" and "Do these "brand" and "concentration" effects show up better with "high" than with "low" salivating individuals within the same strain of mice?" These two questions

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were tested on the existing set of data. The experimental mice within each treatment group were separated into "high" and "low" salivators, and statistical analysis with respect to "brand" and "concentration" was performed on "high" and "low" salivating mice, immediately after and one hour after smoke exposure. The size of the F-values for "brand" and "concentration" will determine the best combination of experimental conditions.

The next slide shows a table with calculated F-values under each of the four experimental conditions. Note first of all, that the concentration effect is much more important to salivary inhibition than the effect of brand. This indicates, among others, that for valid brand comparisons the smoke concentrations have to be standardized rigidly. Note further that highly significant "concentration" effects can be detected under any of the four experimental conditions, but in particular so, when high-salivators are used. On the other hand, any differences between brands were totally undetectable immediately after exposure with high-salivating mice, but they do approach statistical significance one hour after exposure with high-salivating mice.

These results indicate that the salivary inhibitions of high-salivating individuals of a high-salivating strain of mice measured one hour after exposure is optimal to measure any differences in the effects of whole smoke concentrations and brands.

In summary:

A new salivation method in mice was employed to study the effects of various dilutions of whole smokes from two brands of cigarettes. Whole cigarette smokes exert an inhibitory effect on salivation under our experimental conditions, the degree of which is determined by the dose and the brand used. A brand with high acute toxicity showed high salivary inhibition, and a brand with low acute toxicity, low salivary inhibition. Thus a relationship was found between acute toxicity on the one hand and salivary inhibition at pharmacological concentrations on the other hand. Some experimental conditions were examined to detect this relationship most prominently. It is suggested that salivation inhibition by cigarette smoke at pharmacological levels might be predictive for acute toxicity of whole smokes at higher concentrations.

In conclusion:

A relationship in mice exists between inhibition of salivation and acute toxicity of cigarette smoke.

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Source: https://www.industrydocuments.ucsf.edu/docs/lklk0000

Experimental Design To Study the Effects of Four Dilutions of Whole Smokes from Two Cigarette Brands on the Salivary Performance of Male C57BL/6J Mice

Treatment	Dil.	No. of mice	Measurement				
			1	2	3	4	5
no machine exp.	--	48					
machine exp.	--	48					
Brand A	1:32.0	24					
" "	1:16.0	24					
" "	1:8.0	24					
" "	1:5.4	24					
Brand B	1:32.0	24					
" "	1:16.0	24					
" "	1:8.0	24					
" "	1:5.4	24					

1003112915

Source: https://www.industrydocuments.ucsf.edu/docs/lklk0000

Experimental Designs of Two-Day Experiment, To Study the Effects of Four Dilutions of Whole Smokes from Two Cigarette Brands on the Salivary Performance of Male C57BL/6J Mice

Day	Treatment	Dil.	No. of mice	Measurement				
				1	2	3	4	5
I	no machine exp.	--	6					
	machine exp.	--	6					
	Brand A	1:32.0	6					
	" "	1:16.0	6					
	Brand B	1:32.0	6					
	" "	1:16.0	6					
II	no machine exp.	--	6					
	machine exp.	--	6					
	Brand A	1:8.0	6					
	" "	1:5.4	6					
	Brand B	1:8.0	6					
	" "	1:5.4	6					

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Source: https://www.industrydocuments.ucsf.edu/docs/lklk0000

THE TOXICITY OF VARIOUS DILUTIONS OF WHOLE SMOKE FROM TWO BRANDS OF CIGARETTES

Smoke Dilution	Acute Toxicity			
	Mortality*		Time (Min.)	
	A	B	A	B
1:4.0	(24) 24	(24) 24	9.6	14.5
1:8.0	(24) 0	(24) 0	--	--
Machine-treated	(24) 0	(24) 0	--	--

* Maximal exposure period is 60 minutes.

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Source: https://www.industrydocuments.ucsf.edu/docs/lklk0000

ANALYSIS OF VARIANCE OF EXPOSURE TIMES UNTIL DEATH, WHEN MALE C57Bl/6J MICE ARE TREATED WITH A 1:40 DILUTION OF WHOLE SMOKES FROM TWO CIGARETTE BRANDS

Source	DF	MS	F_{cal}	$F_{95\%}$	$F_{99\%}$
Total	47				
Brand	1	1,131	22.62**	4.09	7.31
Experiment	2	276	5.52**	3.23	5.18
Brand x Experiment	2	27	0.54	3.23	5.18
Error	42	50			

** Significant at the 1% level of probability.

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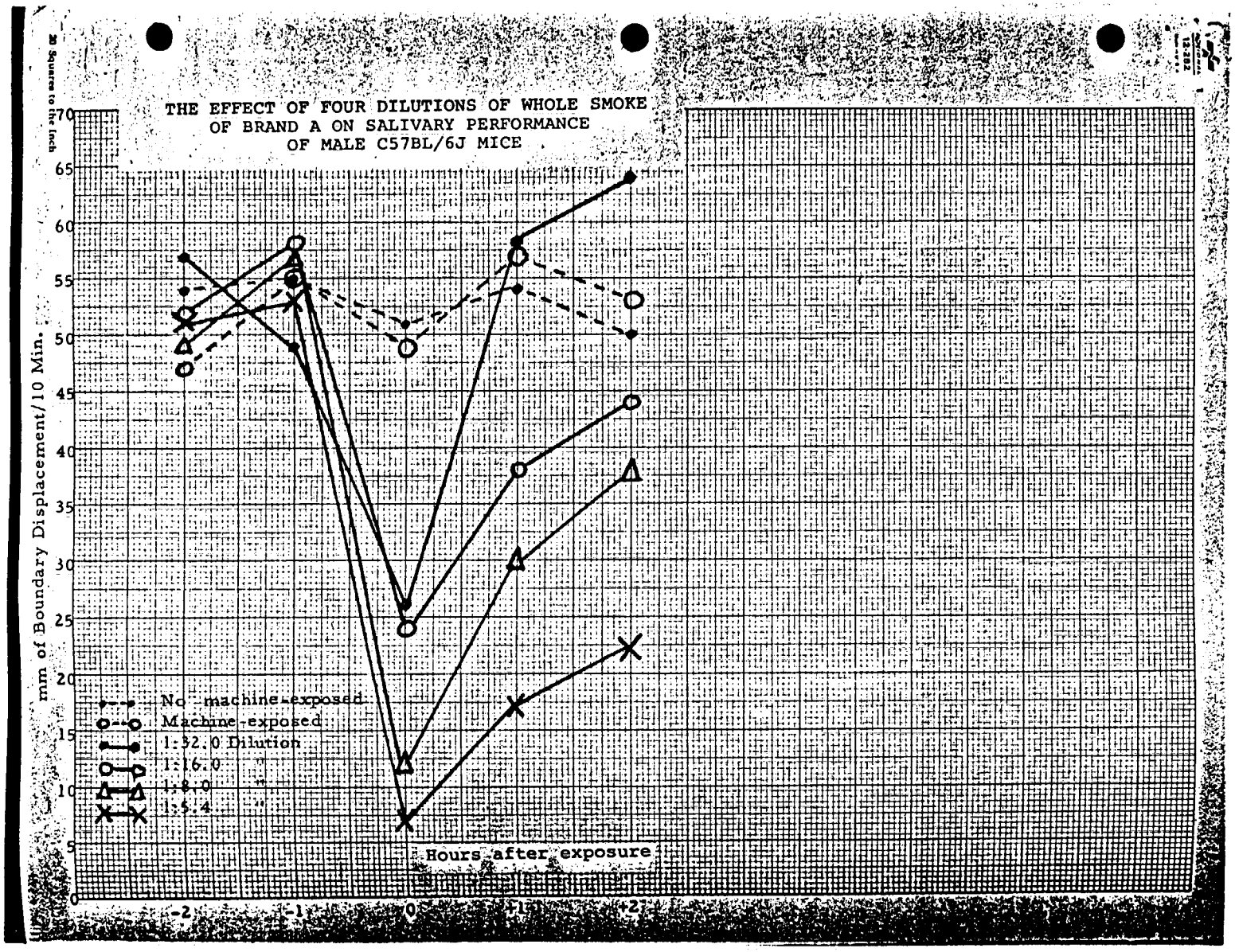
Source: https://www.industrydocuments.ucsf.edu/docs/lklk0000



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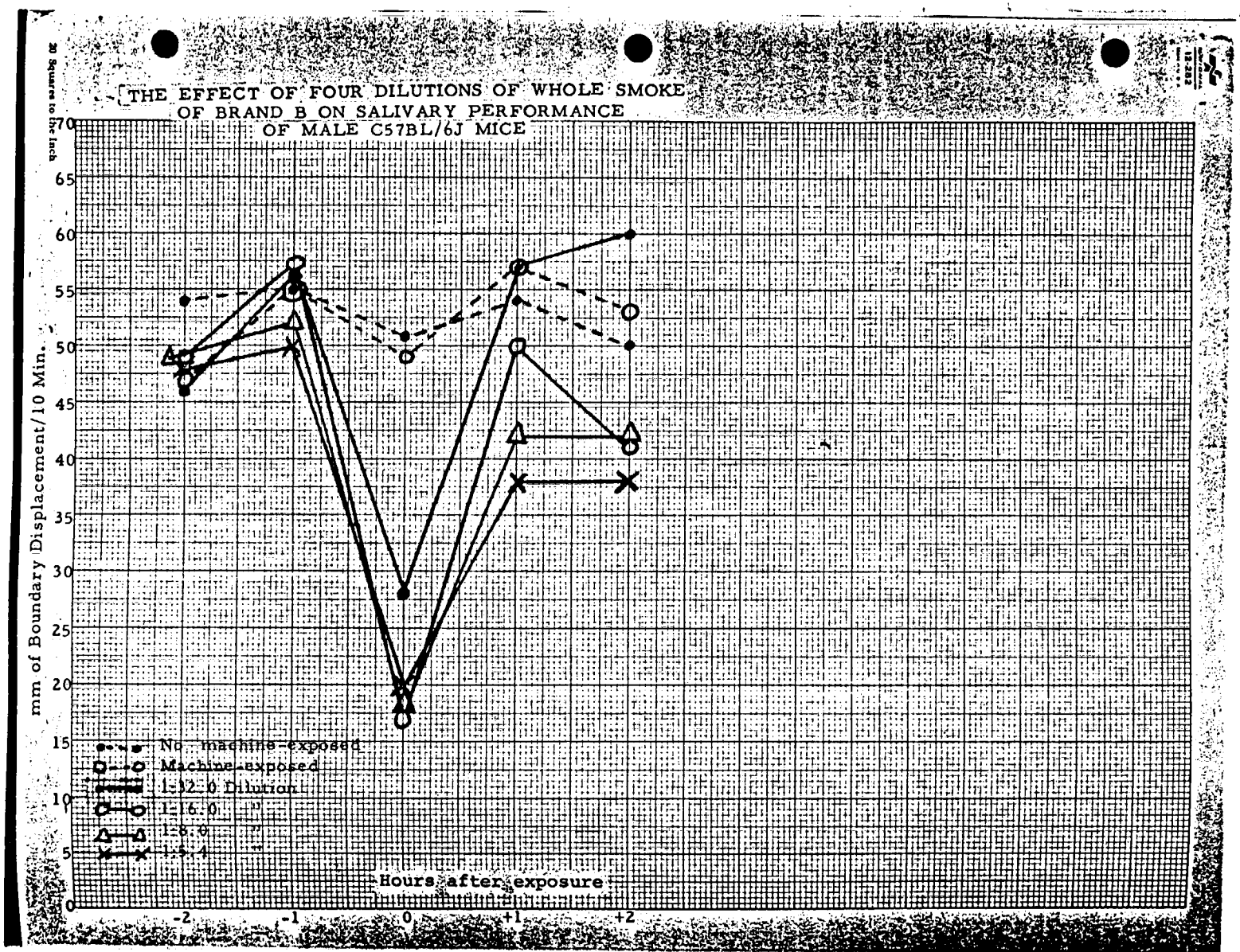

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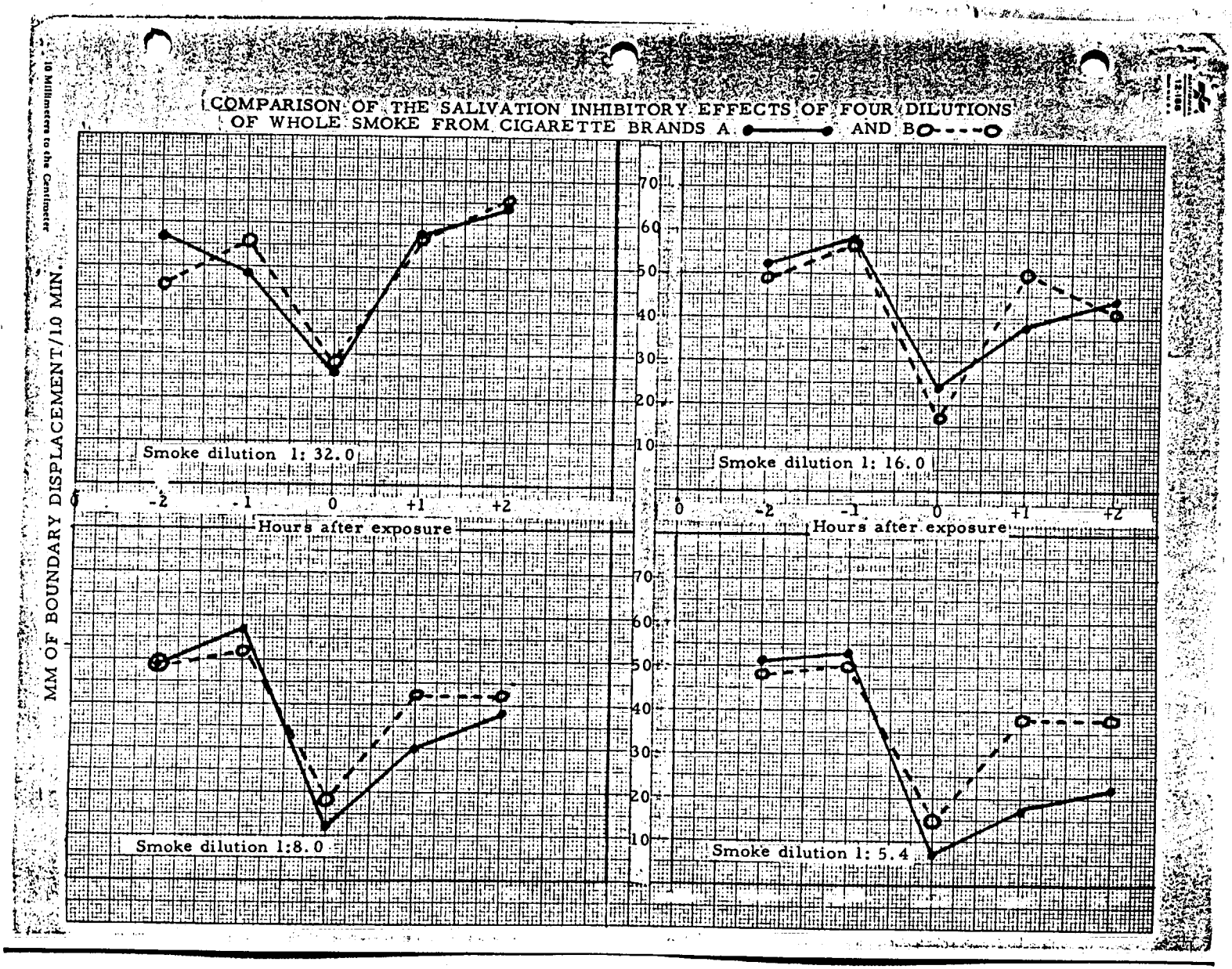

Source: https://www.industrydocuments.ucsf.edu/docs/lklk0000

THE CALCULATED F-VALUES FOR BRAND AND CONCENTRATION, OBTAINED WITH VARIOUS GROUPS OF MICE, AT VARIOUS TIMES AFTER EXPOSURE

Time after exposure (hours)		All		High Sal.		Low Sal.	
		Brand	Conc.	Brand	Conc.	Brand	Conc.
0	F_{cal}	.64	27.30**	1.51	22.82**	0.01	8.95**
1	F_{cal}	3.91*	8.24**	3.64	8.26**	1.11	3.51 **
	$F_{95\%}$	3.84	2.21	3.84	2.21	3.84	2.21

* Significant at the 5% level of probability
** Significant at the 1% level of probability

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Source: https://www.industrydocuments.ucsf.edu/docs/lklk0000

A NEW PARAMETER FOR THE QUANTITATIVE MEASUREMENT OF THE TOXICITY OF CIGARETTE SMOKE*

by

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Bio-Research Institute
Cambridge, Mass. 02141

* Paper presented at the meeting of the American Industrial Hygiene Association in Toronto, Canada, May 24-28, 1971.

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Source: https://www.industrydocuments.ucsf.edu/docs/lklk0000

Parameter of the toxicity of cigarette smoke - Van Dongen

Mr. Chairman, Members of the Society:

The measurement of the toxicity of cigarette smokes is most commonly done with the all-or-non response of death, and the so-called LD_{50} is one of the more popular expressions among them.

The use of an all-or-non response in predicting the toxicity of treatments has at least two drawbacks. First, it is inherently less efficient than a graded response; and second, the experiments are terminal to the animals and thus expensive.

The above-mentioned reasons suggest the need for indirect toxicological methods, whereby a pharmacological response rather than lethality is used. Such a pharmacological response, however, should be related to toxicity and its measurement should be quantitative and simple.

The recently published salivation method in mice, developed by us, meets the above-stated requirements (fall meeting ASPET, 1969). Not only is the method non-traumatic to the animals, quantitative and simple, but it also accommodates the measurement of both direct and after-effects to treatment, and it permits the measurement of salivary performance on large numbers of mice simultaneously. This last feature makes it a suitable screening tool. Finally, it was established that the exposure of mice to cigarette whole smokes inhibits salivary secretion and that the extent of salivary inhibition is positively related to acute toxicities on the basis on experimental evidence with brands and smoke concentrations.

Although the exposure to cigarette smoke generally results in a salivary inhibition, the effect was, however, by no means consistent and reproducible. And since reproducibility is essential for any good methodology, it is the aim of this paper to examine the effect of a variety of factors on the salivary performance of mice; the results of which might aid in establishing improved experimental conditions to obtain consistent salivary inhibitions, predictive for acute toxicity of cigarette smoke.

The specific purpose of this study, therefore, is to examine the following factors of salivary inhibition: (a) whole smokes and gasphases, (b) high salivating and low salivating individuals of the same strain of mice, (c) a wide and narrow dilution range, and (d) the time after exposure.

The method to measure salivation in mice will be shown now.

Slide I shows a mouse in a stanchion by its permanently-worn collar. The mouse bits on a wire bit. A narrow cotton strip, pretreated

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Source: https://www.industrydocuments.ucsf.edu/docs/lklk0000

with pH indicator, is attached to the bit and turns from yellow to purple upon exposure to saliva. The wetted distance of the colored band is measured in mm and represents the amount of saliva excreted.

The next slide shows that a large number of mice can be studied simultaneously. Significant differences in salivary performance exist among various strains of mice. High-salivating C57BL mice. Low-salivating AKR mice.

To perform the salivation experiments, a large herd of male C57BL mice was available. The animals were approximately 24 weeks old, had been housed for some time six to a cage with their collars on. They had been adapted previously to the salivation procedure and were all in good health.

The salivary performance of mice was determined five times with a one-hour interval between measurements. The first and second measurements served to establish the control salivary performance of individual mice during that particular day. A ten-minute smoke exposure was applied immediately prior to the third measurement which will thus indicate the immediate effect to exposure. The fourth and fifth measurements were performed to examine any after-effects to treatments.

Smoke exposures were applied with the Walton-reverse smoking machine. The conditions in the smoking machine were standardized to 35 mm puffs of two seconds duration taken at intervals of 58 seconds. The smoke would persist for 15 seconds in the inhalation chamber and then be expulsed in 3 seconds followed by a fresh air purge for 40 seconds. Ten puffs were studied in each case, using the first five puffs of each of two sets of cigarettes, smoked without interruption in the one puff per minute cycle.

In the gasphase experiment, a cambridge filter was inserted between the proximal end of the cigarettes and the inhalation chamber.

The following ten treatments were applied: no machine exposure, machine exposure with air, or any one of four smoke dilutions from two cigarette brands A or B. The smoke dilutions were 1 to 5.4, 1 to 8.0, 1 to 16 and 1 to 32.0.

The cigarette brands A and B were selected on the basis of their tar content, nicotine content, and acute toxicities. Brand A has a high tar and high nicotine content, along with high acute toxicity. Brand B has a low tar, low nicotine content, along with low acute toxicity. The cigarettes were equalized in length to 65 mm and climatized at 60% relative humidity in a descicator for at least several days until immediately prior to their actual use in the smoking machine.

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Source: https://www.industrydocuments.ucsf.edu/docs/lklk0000

The next slide shows the experimental layout of a two-day experiment with whole smokes on 72 mice. On the first day, the experiment started with determing a first and second salivation measurement on 36 mice. These mice were then rankordered according to salivary performance and systematically assigned to any one of six treatment groups. The various treatments, applied immediately prior to the third salivation are thus done on very equal groups of mice. The second day's experiment on different mice was done the same, except for different smoke dilutions. Such a two-day experiment was repeated four times. Thus this whole smoke study includes approximately 72 x 4 x 5 = 1440 salivation measurements.

The next slide presents the layout of the entire salivation experiment with whole smokes. It is noteworthy that any smoke exposures can be compared validly with two types of controls, namely, first with "no exposure" and "machine exposure only," but second also with their own salivary performances during the first and second measurements since it had been shown that non-significant differences in the salivary performance of mice exist during the day.

The experimental design just described for whole smoke will be employed identically to study the effect of gasphases on salivary inhibition.

The statistical analysis of data was done primarily using the analysis of variance technique.

The next slide shows the salivary performance of mice upon exposure to various dilutions of whole smokes from brand A. The abcis represents the time for exposure, in hours, and the ordinate the level of salivary performance, expressed in mm of boundary displacement. Note the approximate constancy over time and the non-significant differences between the two controls. These curves represent the salivary performances with 1 to 32, 1 to 16, 1 to 8 and 1 to 5.4 dilutions of whole smokes from brand A. Obviously a very dramatic salivary inhibition which is significantly more pronounced in size as well as in duration with higher smoke concentrations.

The next slide shows the salivary performance of mice upon exposure to whole smokes from brand B. It shows a similar pattern as was seen with brand A, although its dose dependence is less distinct.

The next slide compares the salivary inhibitory effects of brands A vs. b, at each of the four whole smoke dilutions. The highest smoke dilution (1 to 32) is represented in the upper left figure. The lowest (1 to 5.4) in the lower right figure.

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Source: https://www.industrydocuments.ucsf.edu/docs/lklk0000

Comparison of the four figures shows clearly the increased salivary inhibition with increased smoke concentrations for each brand. Any systematic, differential inhibition between brands A and B is dilution dependent. There is no difference between the brands at the 1 to 32 dilution. An inconsistently higher salivary inhibition of brand A vs brand B was found at the 1 to 16 dilution, but consistently higher salivary inhibitions by brand A vs B were obtained with th 1 to 8.0 and the 1 to 5.4 dilutions.

Note further that the inhibitory after-effects to whole smoke exposures can extend over a period of several hours, which is quite different from what we will see later on with gasphase exposures.

The next slide summarizes the statistical reliability of the just shown results. From the list of factors that could have an effect on salivary performance, the following were found to be highly significant: smoke concentration, time after exposure, and the "concentration x brand" and "concentration x measurement" interactions.

We will now present the results from the gasphase experiments.

The next slide shows the effect on salivary performance of four dilutions of gasphases from brand A. The salivary inhibition is less pronounced in degree and duration and also not so obviously dose-related, relative to what was seen with whole smoke previously.

The next slide shows the salivation-inhibitory effects to gasphases from brand B. Again, less dramatic than its whole smoke counterpart.

The next slide compares the four gasphases of both brands at four concentrations. It shows that the salivary inhibitions are less pronounced than those with whole smokes, that they are not clearly dose-dependent, and that no systematic differences in salivary inhibition can be observed between the two brands.

Thus, from the standpoint of predicting the toxicity of tobacco smokes, the use of gasphases should be regarded as inadequate, and we will exclude gasphase data, therefor, from further consideration.

We will now focus our attention on the aspects which deal with the salivation level of individual mice, minimal smoke concentration, and optimal time after exposure for salivation measurement. These questions will be examined and statistically tested on the existing set of data.

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The experimental mice within each treatment group were separated into those with "high" and those with "low" salivary performance on the basis of their second measurement.

The next slide shows the salivary performance of "high" vs "low" salivating individuals upon exposure to any one of four concentrations of whole smoke from brand A. Note that the salivary performance of low salivators is affected only to a minor degree. On the other hand, the high salivators show dramatic salivary inhibitions, the degree and duration of which are related to the smoke concentration applied.

The next slide shows the salivary performance data for high vs low salivators upon exposure to whole smokes from brand B. Note the approximate pattern of effects as was observed with brand A.

Thus the experimental evidence with two brands indicates that high salivators are more suitable than low salivators to predict toxicity on the basis of salivary inhibition.

The next slide shows the degree of success in differentiating between whole smoke from brand A and brand B on the basis of salivary inhibition of high salivators. There is no differential salivary inhibition between the brands at the 1 to 32 dilution. The 1 to 16 dilution appears to be the threshold whereby whole smoke from brand A becomes more inhibitory than that from brand B. There is a clearcut differential salivation-inhibitory effect between brands A and B with the two highest smoke concentrations.

With respect to time after exposure, to measure salivary inhibition for predictive purposes of toxicity, the slide suggests that one-hour after exposure is most optimal.

A statistical analysis was performed to examine the validity of the various differences shown graphically. The size of the F-values obtained for "brand" and "dilution" will determine the best combination of experimental conditions.

The next slide shows a table with calculated F-values under each of the 12 experimental conditions. A glance at the figures indicates the presence of larger F-values with high than with low salivators. Note further that, except for one, all are higher when the highest dilution is excluded. On the basis of dilution alone, 0, 1 or 2 hours after exposure would all be significant. However, the additional requirement to differentiate among brands narrows the optimal hour after exposure down to

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one at 1 hour. Note further that the dilution effects are, under our experimental conditions, much more important to salivary inhibition than the effect of brand. This indicates, among others, that for valid brand comparisons, the smoke concentrations have to be standardized rigidly.

In Summary:

C57BL mice were exposed to four dilutions of whole smokes or gasphases from two brands of cigarettes and their salivary performance was determined immediately after, and one and two hours after exposure. Brand A has high tar and high nicotine content along with a high acute toxicity, and brand B has a low tar and low nicotine content along with low acute toxicity.

The predominant effect of the exposures to whole smokes and gasphases was that of a salivary inhibition. The salivary inhibition is more reproducible and more pronounced with whole smokes than with gasphases. To predict toxicity of cigarette whole smokes, high-salivating mice should be exposed to smoke concentrations of 1 to 16 or higher, and salivary performance should be measured one hour after exposure.

The establishment of these experimental conditions suggest that the degree of salivary inhibition can now be used to predict acute toxicity of cigarette smokes

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EXPERIMENTAL DESIGN OF TWO-DAY EXPERIMENT TO STUDY THE EFFECTS OF FOUR DILUTIONS OF WHOLE SMOKES FROM TWO CIGARETTE BRANDS ON THE SALIVARY PERFORMANCE OF MALE C57BL/6J MICE

Day	Treatment	Dil.	No. of Mice	Measurement				
				1	2	3	4	5
I	No machine exp.	--	6					
	Machine exp.	--	6					
	Brand A	1:32.0	6					
	" "	1:16.0	6					
	Brand B	1:32.0	6					
	" "	1:16.0	6					
II	No machine exp.	--	6					
	Machine exp.	--	6					
	Brand A	1:8.0	6					
	" "	1:5.4	6					
	Brand B	1:8.0	6					
	" "	1:5.4	6					

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EXPERIMENTAL DESIGN TO STUDY THE EFFECTS OF FOUR DILUTIONS OF WHOLE SMOKES FROM TWO CIGARETTE BRANDS ON THE SALIVARY PERFORMANCE OF MALE C57BL/6J MICE

Treatment	Dil.	No. of Mice	Measurement				
			1	2	3	4	5
No machine exp.	--	48					
Machine exp.	--	48					
Brand A	1:32.0	24					
" "	1:16.0	24					
" "	1: 8.0	24					
" "	1: 5.4	24					
Brand B	1:32.0	24					
" "	1:16.0	24					
" "	1: 8.0	24					
" "	1: 5.4	24					

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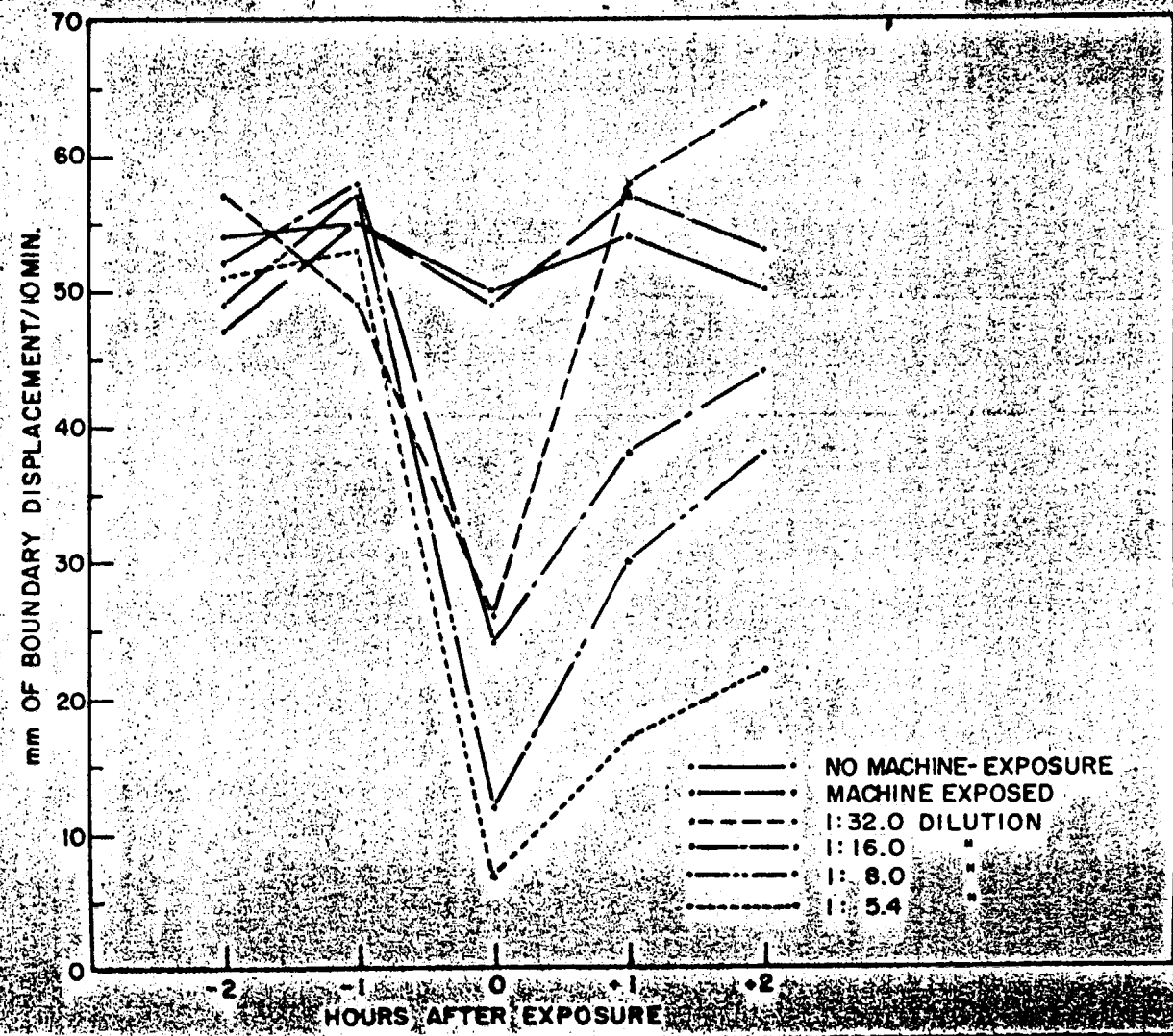
THE EFFECT OF FOUR WHOLE SMOKE DILUTIONS OF BRAND A ON SALIVARY PERFORMANCE OF C57BL/6J MICE



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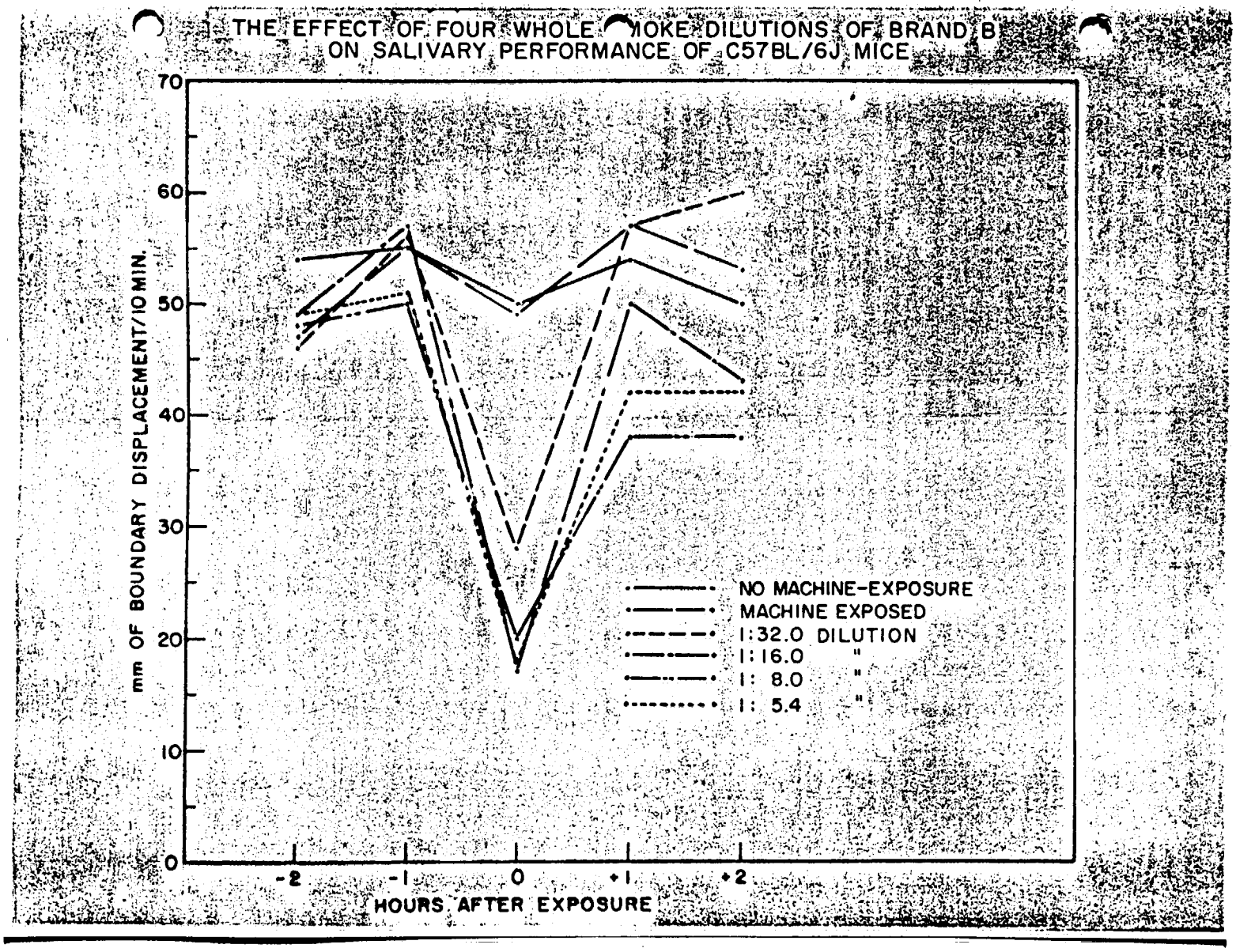

Source: https://www.industrydocuments.ucsf.edu/docs/lklk0000

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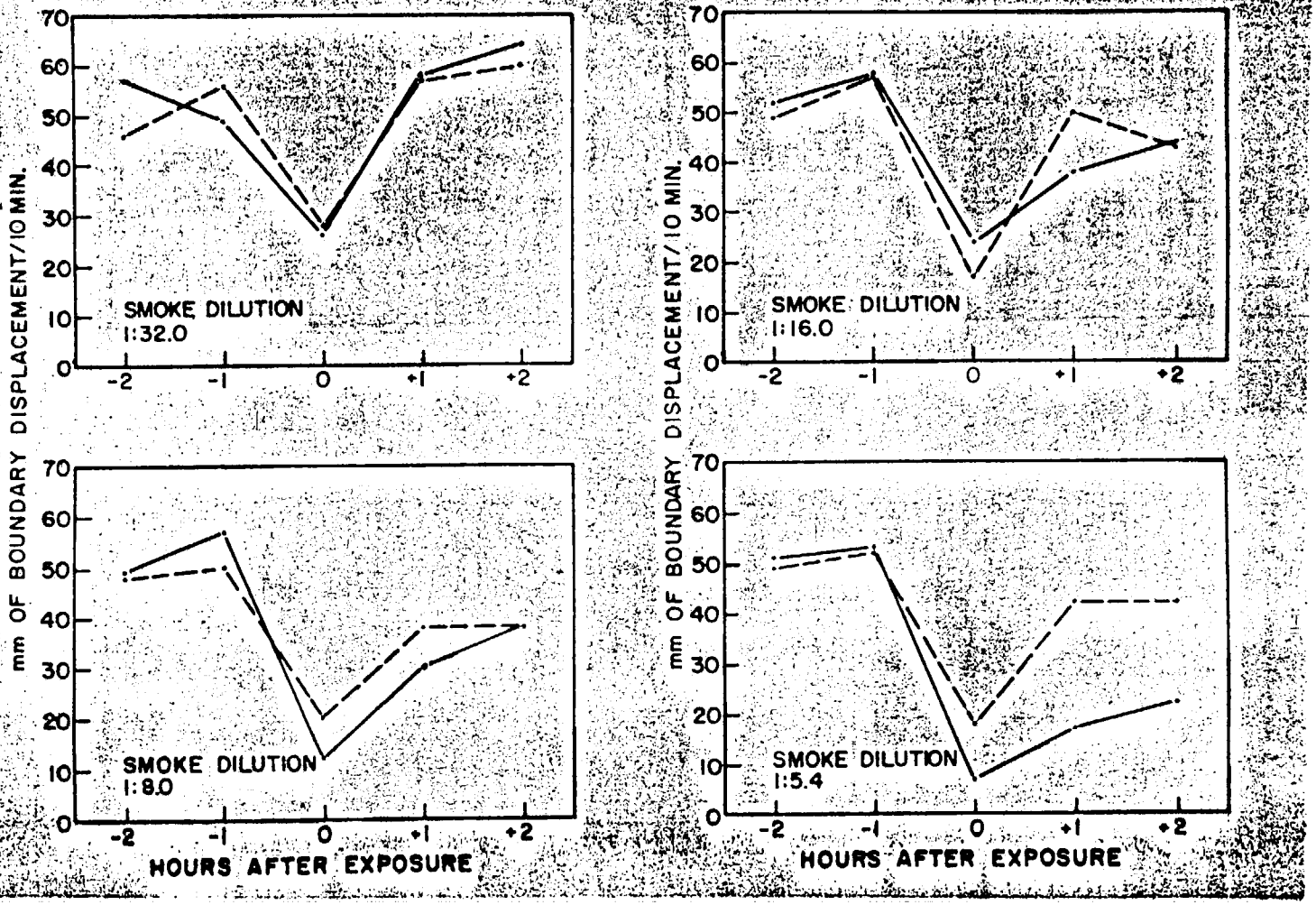
COMPARISON OF THE SALIVARY PERFORMANCE OF C57BL/6J MICE, UPON EXPOSURE TO FOUR DILUTIONS OF WHOLE SMOKE FROM BRANDS A ——• vs B– – –•



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Source: https://www.industrydocuments.ucsf.edu/docs/lklk0000

ANALYSIS OF VARIANCE

The Effects of Various Dilutions of Whole Smokes from Two Brands of Cigarettes on the Salivary Performance of Male C57BL/6J Mice (0, 1, and 2 hours after exposure)

Source	DF	MS	F_{cal}	$F_{95\%}$	$F_{99\%}$
Total	815				
Brand(B)	1	1,095	1.53	3.84	6.64
Concentration(C)	5	20,159	28.08**	2.21	3.02
Measurement(M)	2	26,201	36.49**	3.00	4.61
B x C	5	2,589	3.61**	2.21	3.02
B x M	2	344	0.48	3.00	4.60
C x M	10	2,143	2.98**	1.94	2.51
B x C x M	10	553	0.77	1.94	2.51
Error	780	718			

** significant at the 1% level of probability

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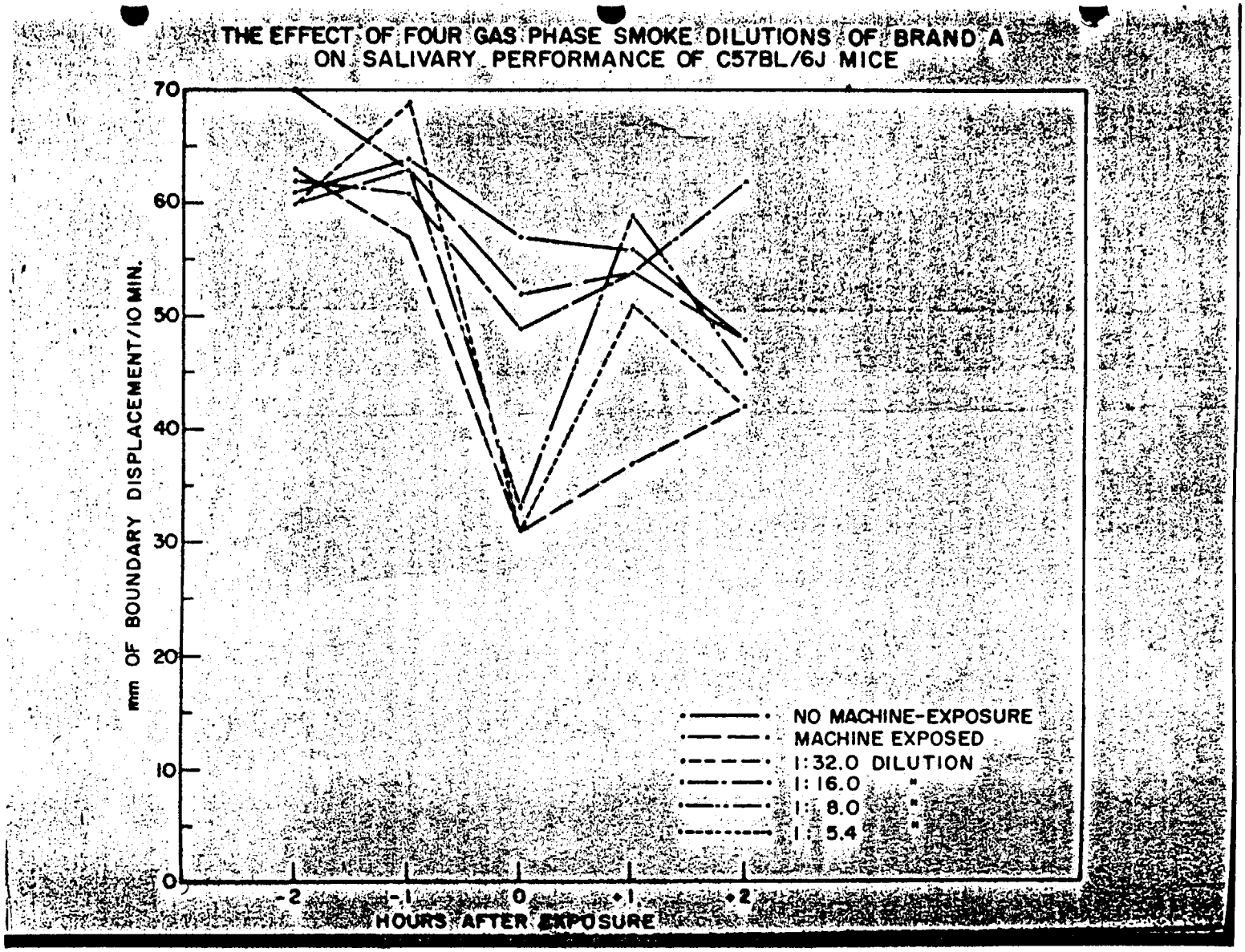
Source: https://www.industrydocuments.ucsf.edu/docs/lklk0000



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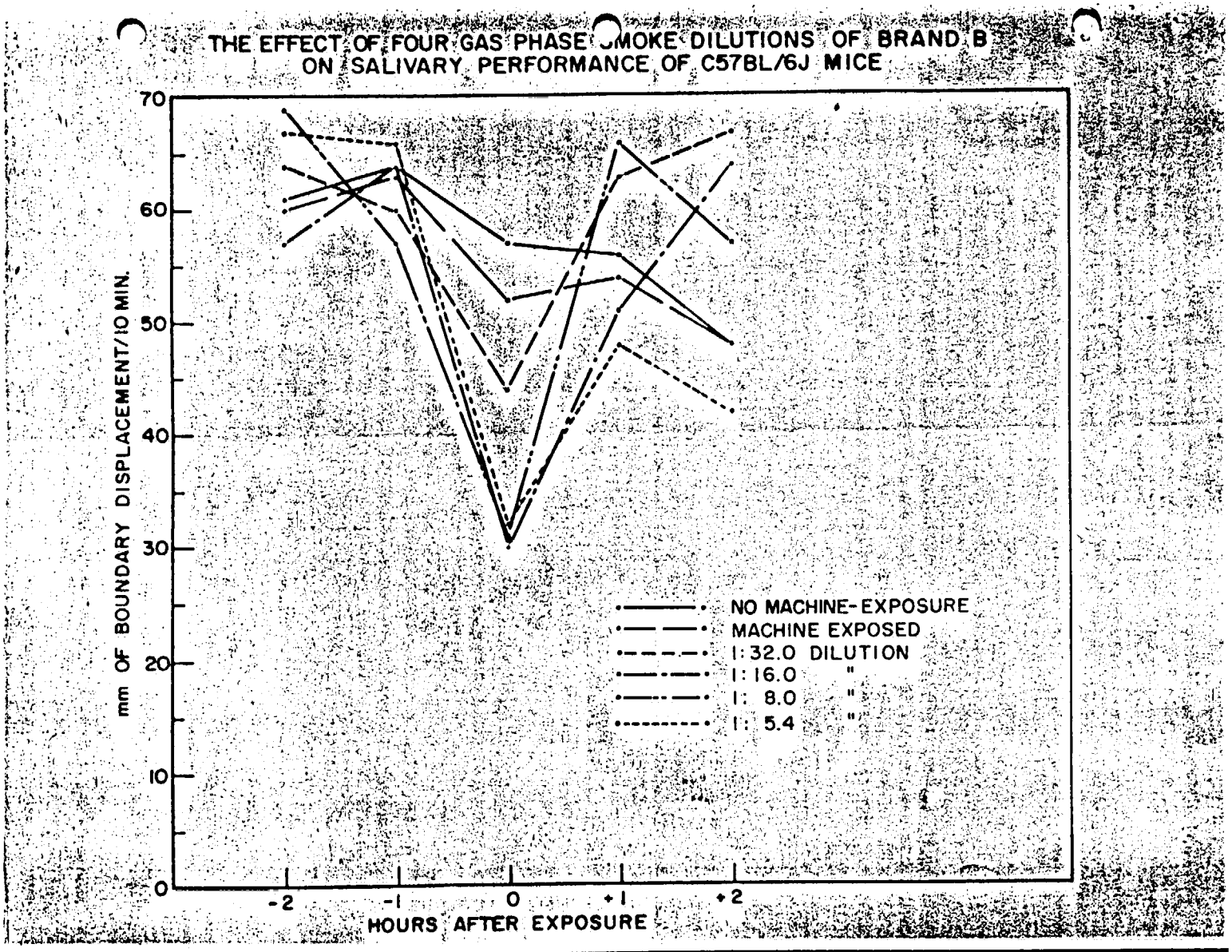

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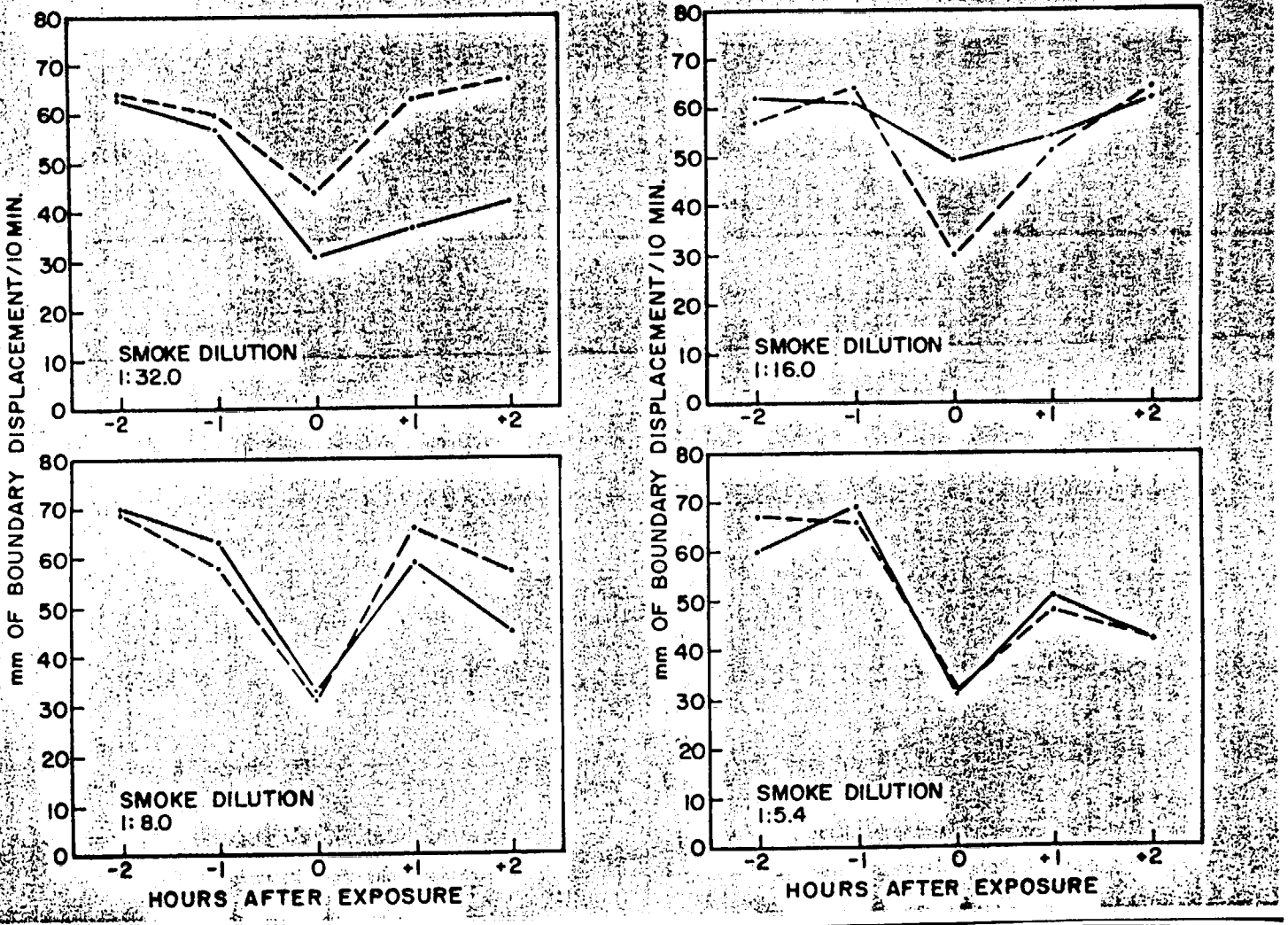
Source: https://www.industrydocuments.ucsf.edu/docs/lklk0000

COMPARISON OF THE SALIVARY PERFORMANCE OF C57BL/6J MICE UPON EXPOSURE TO FOUR DILUTIONS OF GAS PHASE SMOKE FROM BRANDS A ——• vs B •——



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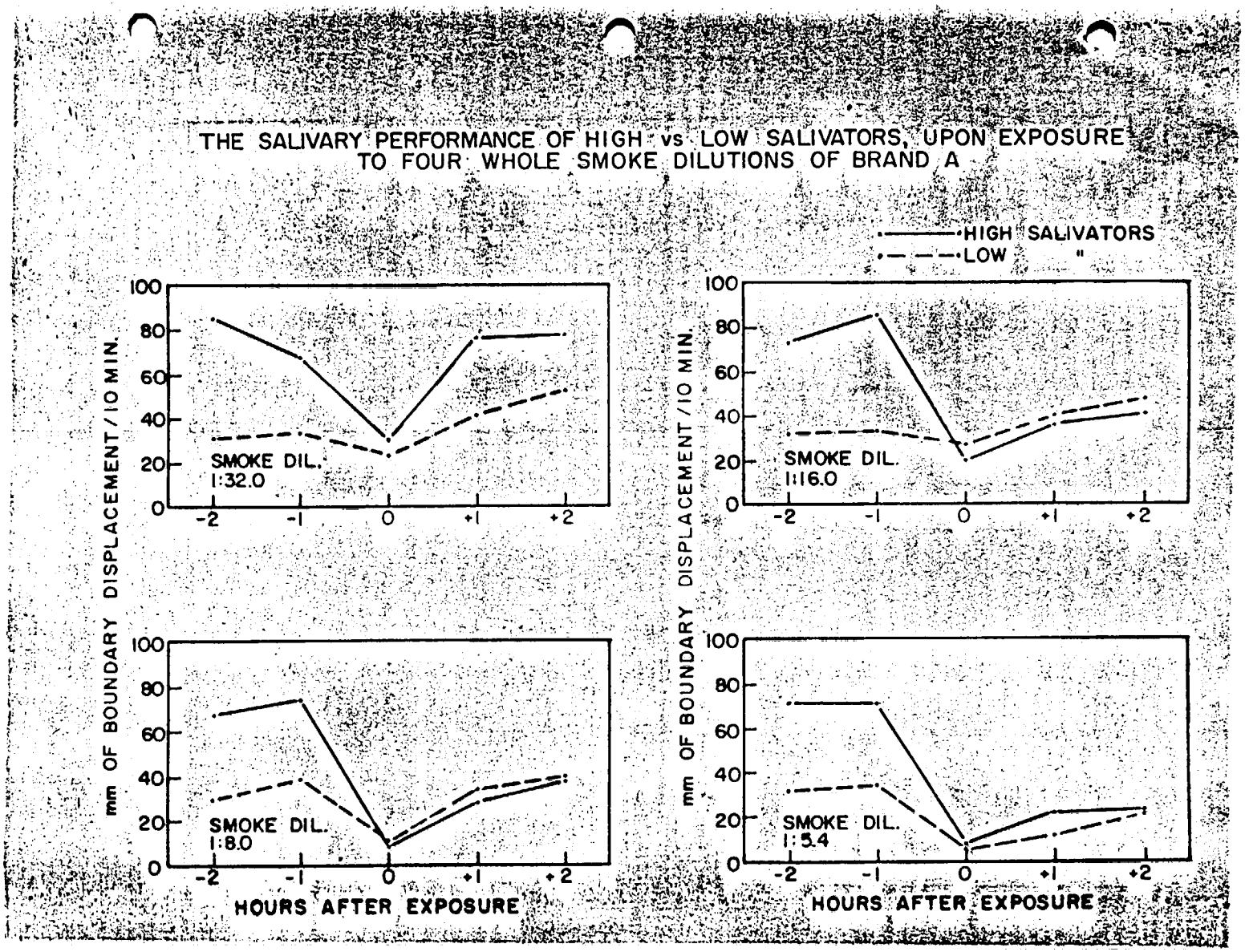
Source: https://www.industrydocuments.ucsf.edu/docs/lklk0000



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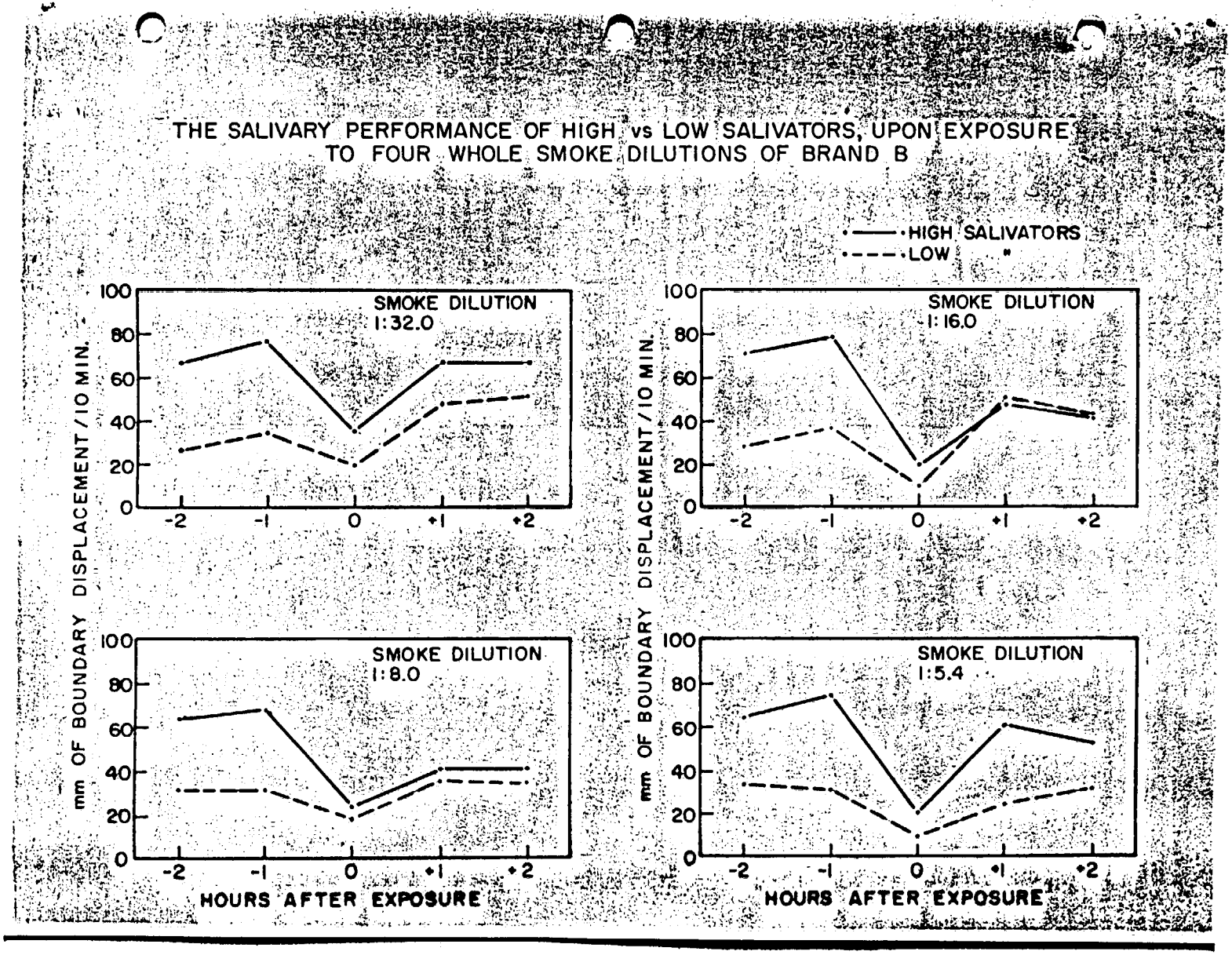

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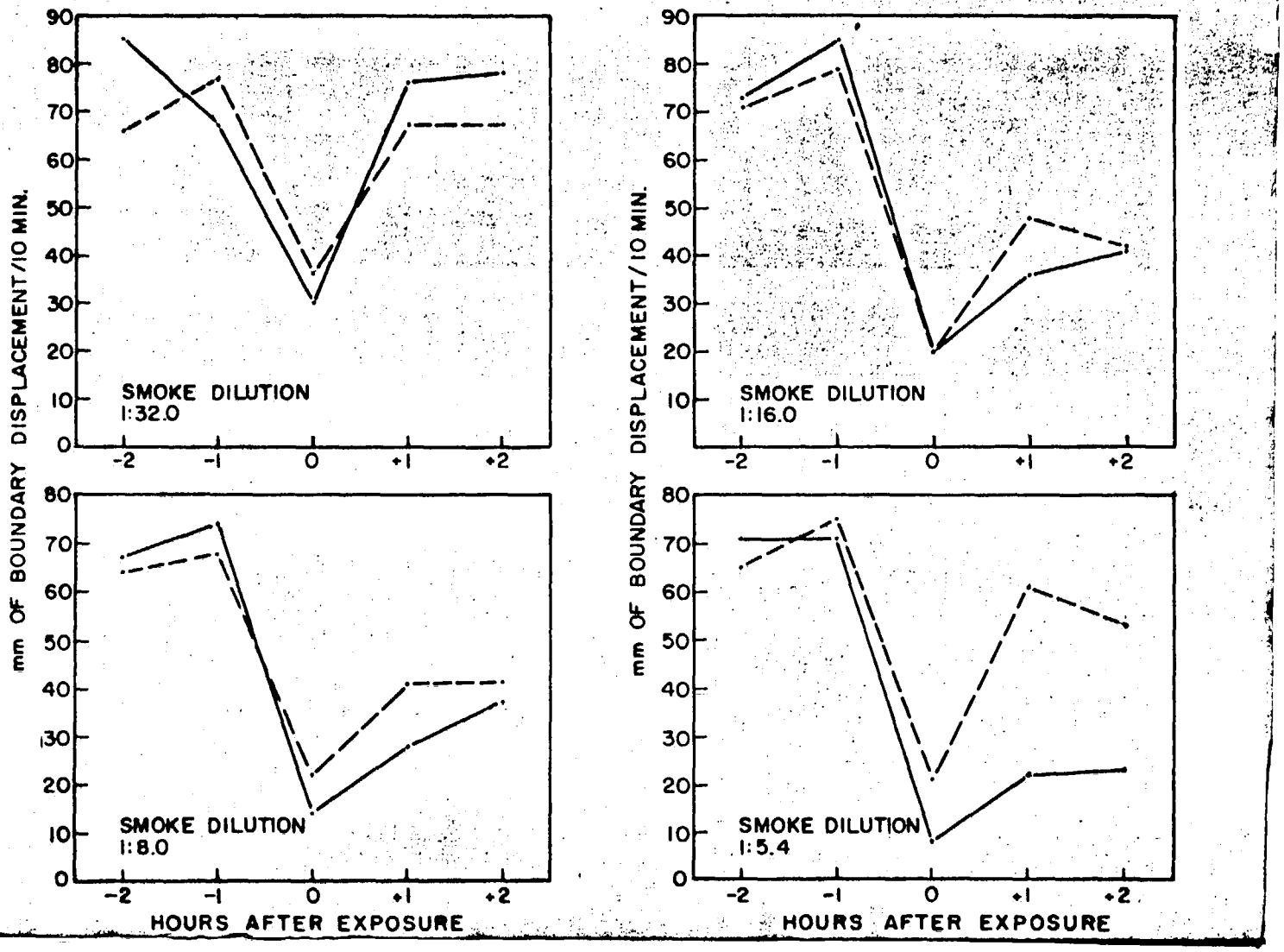
Source: https://www.industrydocuments.ucsf.edu/docs/lklk0000

COMPARISON OF THE SALIVARY PERFORMANCE OF HIGH-SALIVATING C57BL/6J MICE UPON EXPOSURE TO FOUR DILUTIONS FROM BRANDS A —— vs B - - -



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Source: https://www.industrydocuments.ucsf.edu/docs/lklk0000

THE CALCULATED F-VALUES FOR "BRAND" AND "DILUTION" OBTAINED WITH "HIGH" AND "LOW" SALIVATORS AT THREE DIFFERENT TIMES AFTER EXPOSURE TO A WIDE OR NARROW DILUTION RANGE OF WHOLE SMOKES

Parameter	Time after Exposure (hours)	High Salivators		Low Salivators	
		6 Dil.	4 Dil.	6 Dil.	4 Dil.
"Brand"	0	1.51	1.21	0.01	0.00
	1	3.64	5.63*	1.11	0.79
	2	0.79	1.76	0.00	0.00
"Dilution"	0	22.82**	37.44**	8.95**	13.99**
	1	8.26**	9.71**	3.51**	5.33**
	2	5.39**	4.25**	2.19	2.36

* Significant at the 5% level of probability

** " " " 1% " " "

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Source: https://www.industrydocuments.ucsf.edu/docs/lklk0000

BIPHASIC RESPONSE OF SALIVARY PERFORMANCE IN MICE FOLLOWING NICOTINE TARTRATE*

by

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Cambridge, Mass. 02141

* Paper to be presented at the 55th annual FASEB meeting, to be held in Chicago, Ill., on April 12-17, 1971.

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Source: https://www.industrydocuments.ucsf.edu/docs/lklk0000

Mr. Chairman, Members of the Society:

We reported previously on a new method for the quantitative measurement of salivation in mice. The method is based on a sensitive physiological response, is non-traumatic to the animal, does not require anesthesia, provides information on immediate and after-effects to applied treatments, measures quantitative rather than qualitative, allows for simultaneous measurements on large numbers of mice, and is thus suitable for adequate statistical analysis of the experimental data.

Since this methodology possesses certain intrinsic advantages, it is important to make a detailed characterization of the salivary response in this system and to compare wherever possible the obtained results with those published in other species and differing methodologies.

In this respect, we found that various physiological factors such as sex and strain do affect the salivary performance. Exposure to tobacco smokes inhibits salivation. The degree and duration are dependent on the dose. The known "salivation inhibitory drug " atropine-sulfate was found to inhibit the salivation in a dose-related fashion, whereas the known "salivation stimulatory drug" metacholine shows a dramatic and dose-related initial salivary stimulation followed by an equally dramatic salivary inhibition. The dose-response relationship with the two drugs were so striking and reproducible that it was suggested to use the salivary measurement in mice as a biological assay for cholinergic and anticholinergic drug substances.

In an effort to broaden the experimental data base of the mouse salivation method for improved standardization and methodological comparisons, and to aid in the understanding of the control of salivary secretion in the mouse, we have now selected to study the ganglionic blocking agent nicotine. According to the literature, nicotine is known to have a biphasic action on salivation, namely that of an initial stimulation followed by an inhibition. The highest salivary stimulations can be expected with the lowest nicotine dosages. Although this information is generally true, it is well known that extreme variability in response exists from animal to animal, which makes the biphasic salivary response often unreproducible. The underlying physiological causes for this variability are not well understood.

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Source: https://www.industrydocuments.ucsf.edu/docs/lklk0000

The specific objectives of this study are: (1) to confirm or disprove a biphasic response of nicotine on the salivary performance of mice; (2) to determine the dose-dependence of the response; (3) to examine the validity of the above for a "genetically high" and a "genetically low" salivating strain of mice.

The two strains of mice selected for this study were the "high-salivating" C57BL and the "low-salivating" AKR strain, both obtained from the Jackson Laboratories in Bar Harbor, Maine. Of each strain, a large herd of male mice was available.

The first slide illustrates the measurement of salivary performances in various strains of mice: high-salivating C57BL mice; low-salivating AKR mice.

The next slide shows the experimental layout of a single day's experiment with either the C57BL or AKR mice. Salivary performances of 36 mice were determined four times with a one-hour interval between measurements. The first and second measurements served to establish the control salivary performance of individual mice during that particular day. The mice were then rankordered according to salivary performance and systematically assigned to any one of six treatment groups. The various treatments were applied, immediately prior to the third measurement. The fourth measurement was performed to examine any after-effects to treatments. Nicotine tartrate was injected subcutaneously in 0.1 ml of water.

It is noteworthy that any treatment can be compared validly with two types of controls, namely first with "no-injection," and "injection of water," but also second with their own salivary performances during the first and second measurements, as earlier results had shown that non-significant differences in salivation exist during the day.

A single day's experiment was repeated 4 times for each strain. Therefore, the entire study was done on approximately 288 mice and provided 1152 salivation measurements. The salivary responses to nicotine were examined qualitatively and quantitatively.

We will first present some qualitative data.

The next slide shows the frequencies of an increase in salivary performance by individual mice immediately following the applied treatment. More than 50% would indicate an overall increase, and less than 50% a decrease. As you see, the overall immediate response to

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nicotine seems that of slight salivary stimulation. A closer look at the data reveals that increases are more prevalent with low-salivating than with high-salivating animals. This finding holds true among strains and among individual animals within each strain.

The next slide shows the frequencies of salivary increases one-hour after injection. The predominant effect now is that of salivary inhibition, with the possible exception of low-salivating individuals of the C57BL strain.

The qualitative data thus indicate that nicotine increases salivary performance initially, followed by a salivary inhibition, and that this effect shows up most clearly with the low-salivating individuals of each strain.

We will now turn our attention to quantitative observations.

The next slide shows the effect of nicotine tartrate on the salivary performance of the low-salivating AKR mice, which have a base-salivation of approximately 25 mm of boundary displacement. Note first of all, that the control and water injections are approximately stable over time. The injection with nicotine shows an immediate stimulatory or "no effect," the highest stimulation occurring with the lowest dose. The after-effect is that of a salivary inhibition, the degree of which is positive related to the dose.

The next slide shows the salivary performances of high- or low-salivating individuals of the AKR strain in response to one of 4 doses of nicotine tartrate. Note that in low-salivating individuals, salivation is initially increased at all dose levels and is followed by slight decreases when compared with the salivary performance during the second measurement. With the high-salivating AKR's, only the lowest dose of nicotine showed a dramatic initial stimulatory effect, a slight stimulation was noticed with 0.30 mg, whereas 0.10 and 1.00 mg did not show any effect. The after-effect was clearly that of a salivary inhibition which seems to be dose related.

The next slide shows the effect of various doses of nicotine on the salivary performance of the high-salivating C57BL strain, whose mean base-salivation level is approximately 55 mm of boundary displacement. Here again, the two controls are reasonably stable over time. Note further that the most dramatic initial salivary stimulation occurs with the lowest nicotine dose, whereas the initial salivary decreases are obtained with higher nicotine doses. The after-

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effect to nicotine is again a salivary inhibition.

The next slide shows the effect of various doses of nicotine on the salivary performances of low- and high-salivating individuals of this high-salivating strain of mice. In the low-salivators, the immediate response to nicotine was in 3 of 4 dosages that of a stimulation, with the most dramatic one showing up at the lowest nicotine dose. The after-effect was that of slight inhibition. With high-salivators, on the contrary, all nicotine dosages were inhibitory to salivation, both initially and one-hour after injection.

The next slide shows a comparison of the salivary performances of AKR vs. C57BL mice in response to various doses of nicotine tartrate. At the lowest nicotine dose, both strains show a clearcut initial stimulation. With the three higher nicotine doses, slight stimulations can be observed with the AKR's and salivary inhibitions with the C57BL's.

The obtained data with mice support results published in the literature with different species, with respect to the presence of a biphasic response on salivation by nicotine and with respect to its dose-dependence. Our results show that this phenomenon holds in principle true in two different strains of mice, but that quantitatively, the base salivation level of mice is of critical importance. Physiologically, our findings can be explained as follows: salivary performance consists of two components, namely spontaneous salivation and induced salivation. Induced salivation is under direct neural control, both through the sympathetic and parasympathetic nerve systems. Nicotine stimulates the ganglion cells at low concentration. At higher concentration, it blocks them with as a result a prolonged salivary inhibition.

In Summary:

A new salivation method in mice was used to study the effect of various doses of subcutaneously injected nicotine tartrate on the salivary performance of high- and low-salivating individuals from two strains of mice. Nicotine exerts a biphasic response on salivation in mice. To notice these effects, the salivary level of the mice and the dose of nicotine employed are major factors. The strain of mice per se does not seem to exert any influence. The results are in agreement with information to this effect in other species and can be explained with the ganglionic action of nicotine.

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EXPERIMENTAL DESIGN FOR A SINGLE DAY'S EXPERIMENT, STUDYING THE EFFECT OF VARIOUS DOSES OF NICOTINE TARTRATE ON THE SALIVARY PERFORMANCE OF MALE C57BL/J MICE. (INJECTION SUBCUTANEOUSLY AND IMMEDIATELY PRIOR TO THE THIRD MEASUREMENT)

Treatment	No. of animals	Measurement			
		1	2	3	4
No injection	6				
Injection with H_2O	6				
0.03 mg nic. tart.	6				
0.10 mg nic. tart.	6				
0.30 mg nic. tart.	6				
1.00 mg nic. tart.	6				

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Number of Mice That Show an Increased Salivary Performance Immediately after a Subcutaneous Injection with Nicotine Tartrate (When Compared to the Second Salivation Measurement and Expressed as Percentage)

Strain	Salivation Level		Overall
	"Low"	"High"	
AKR/J	70	56	63
C57BL/6J	63	31	47
Combined	66	43	54

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Number of Mice That Show an Increased Salivary Performance One Hour after a Subcutaneous Injection with Nicotine Tartrate (When Compared to the Second Salivation Measurement and Expressed as Percentage)

Strain	Salivation Level		Overall
	"Low"	"High"	
AKR/J	38	29	33
C57BL/6J	62	23	42
Combined	50	25	38

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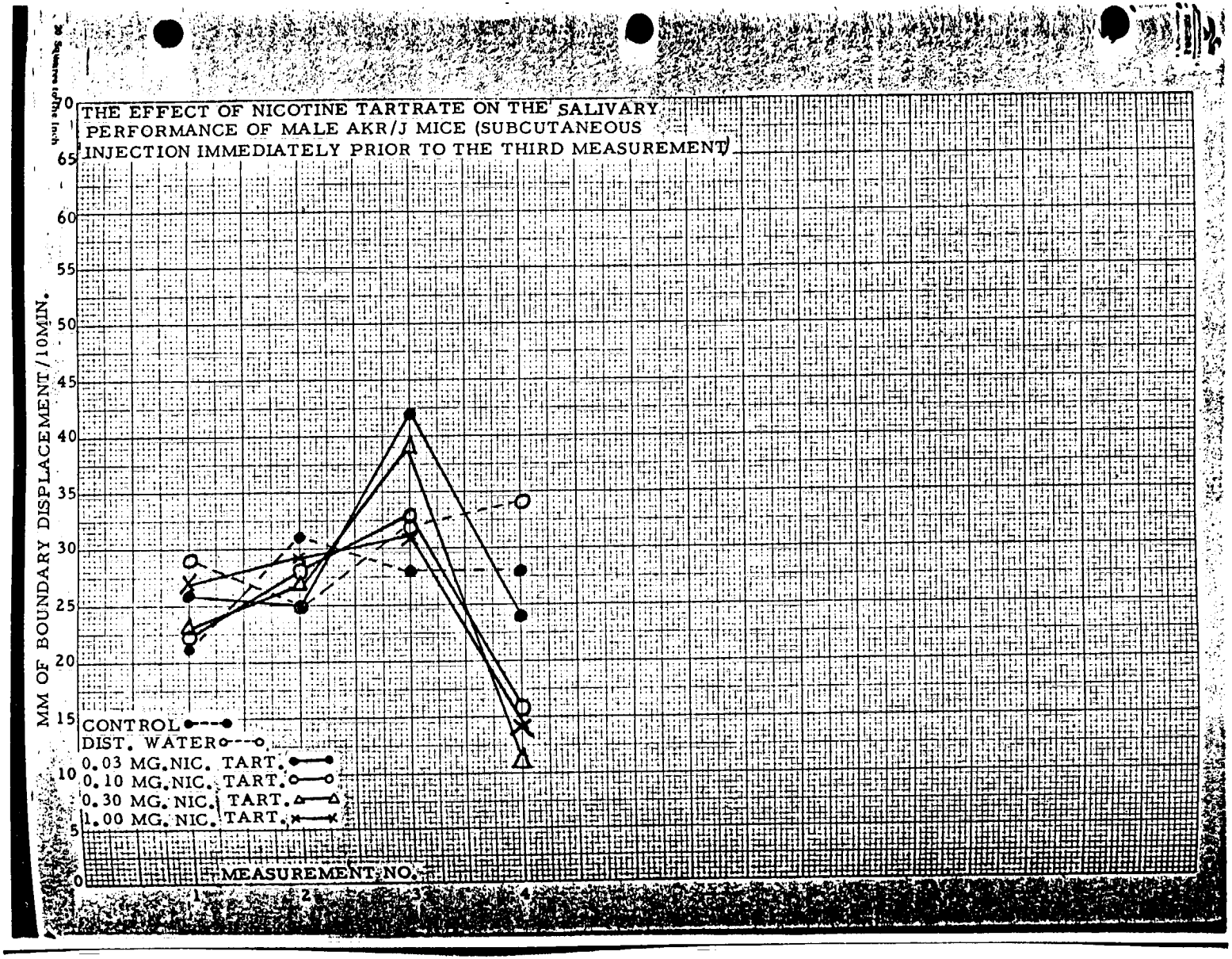

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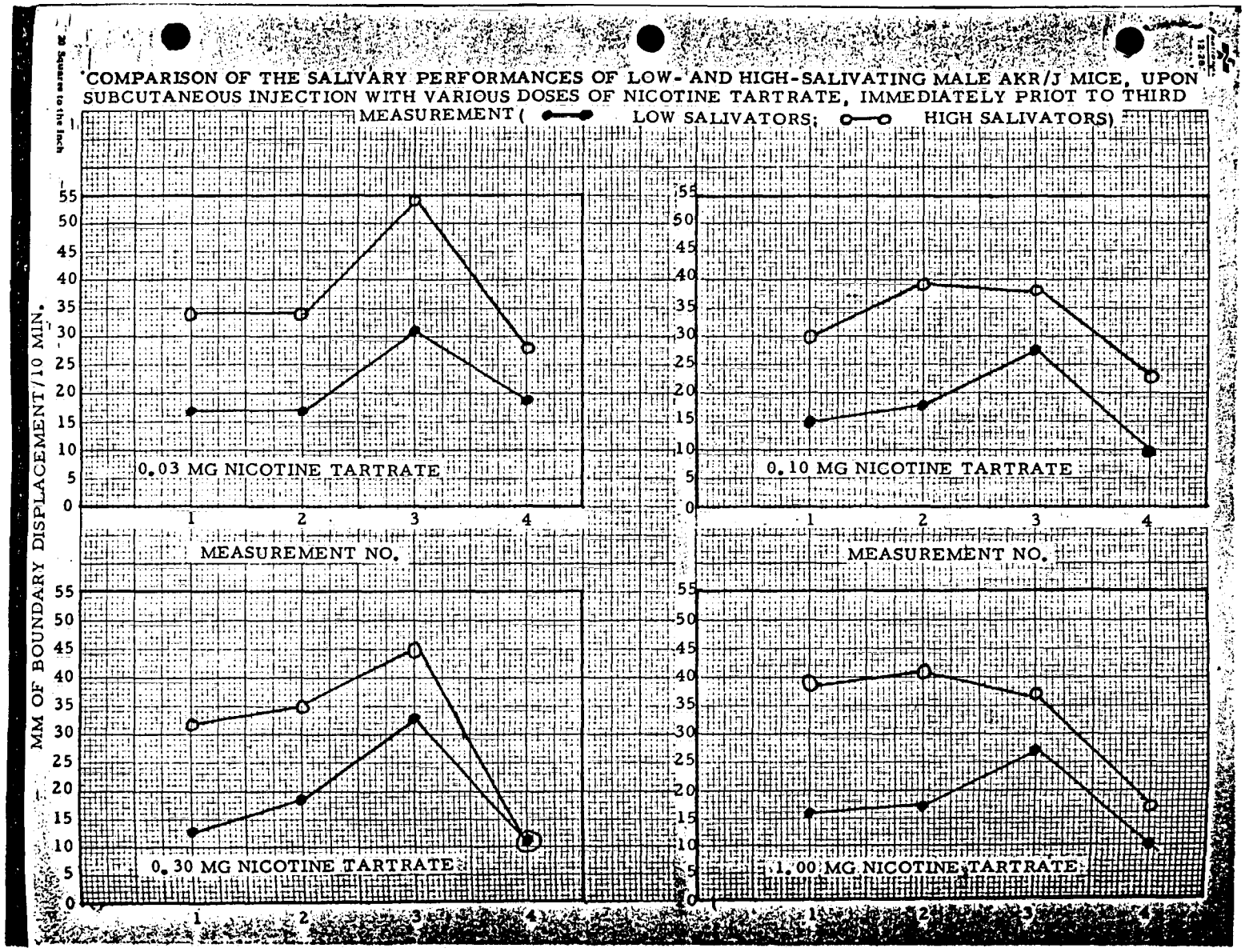

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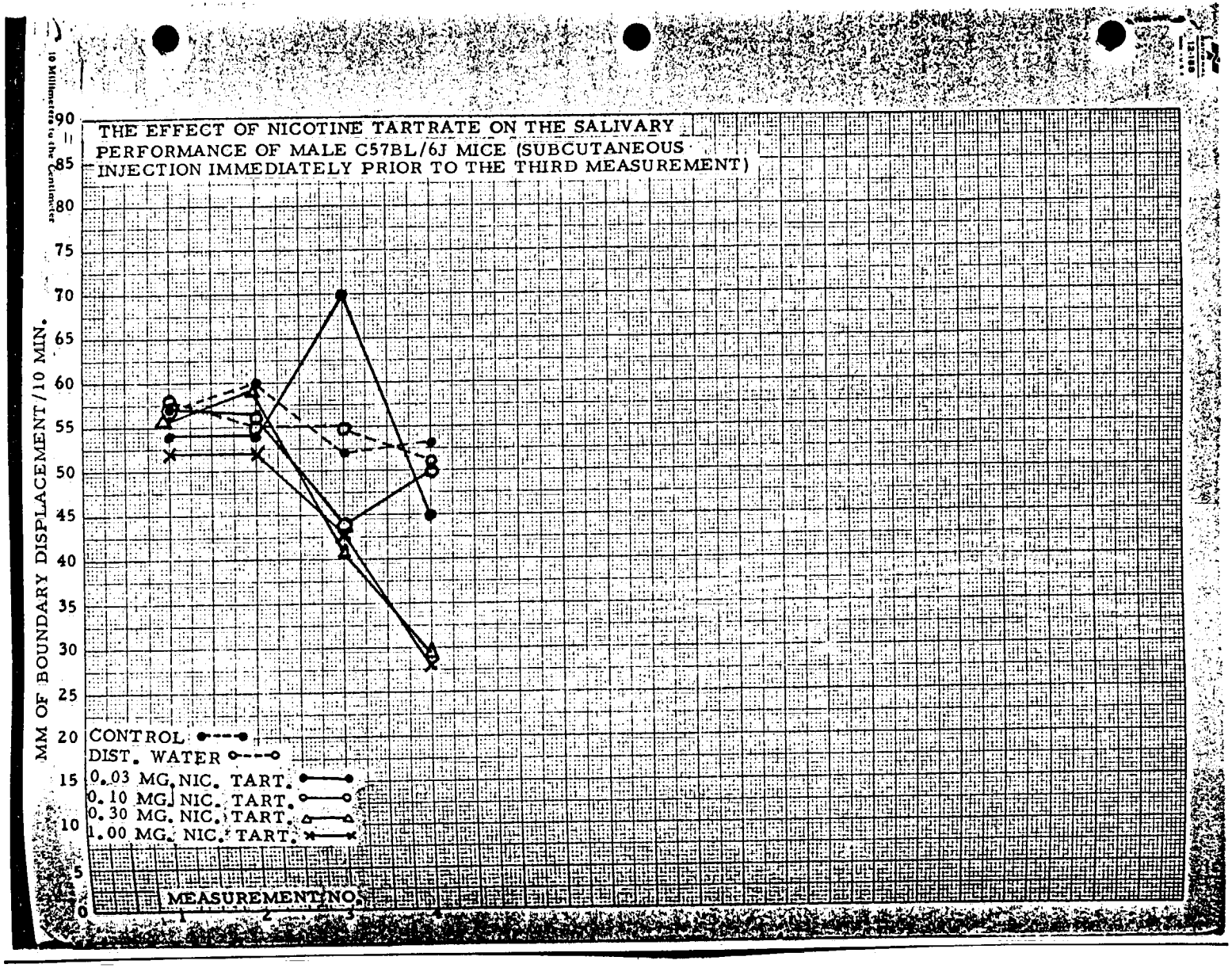

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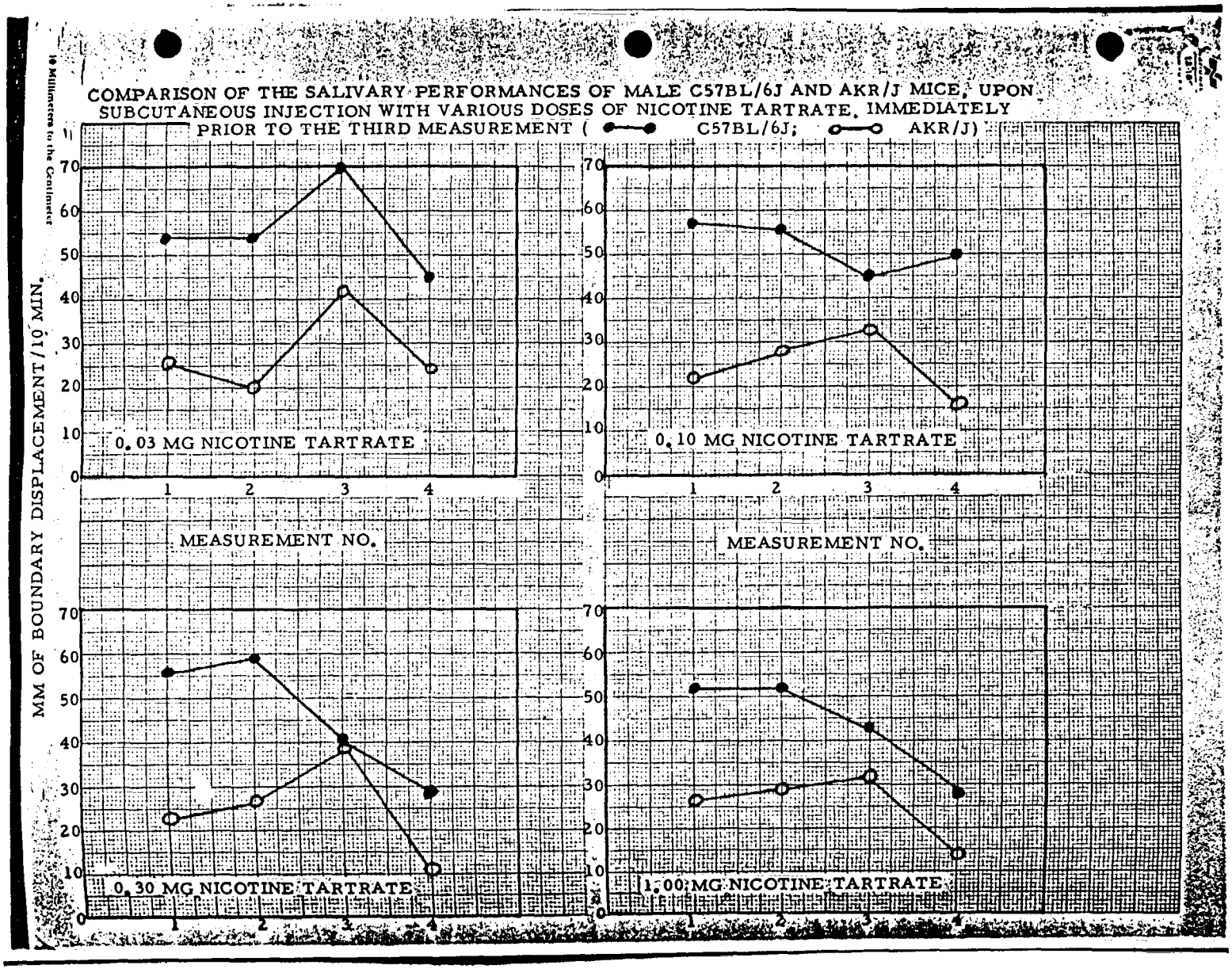

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